Molecular Components Affecting Ocular Carotenoid and Retinoid Homeostasis

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1. Introduction

More than 100 years ago, vitamin A was discovered as a minor but vital lipid growth factor in ordinary foodstuff (McCollum, 1913; Osborne, 1913). Soon after, it was realized that certain yellow plant pigments, named carotenes, possess the same activity (Steenbock, 1919). In 1930, Moore showed that purified carotene was converted to an active growth promoting substance in animal physiology (Moore, 1930). Determination of the chemical structures of β-carotene and retinaldehyde by Paul Karrer and colleagues verified the kinship between the provitamin and its vitamin metabolite (Karrer P, 1930).

A connection between diet and vision was known since ancient times because malnutrition was strongly associated with night blindness (Wolf, 2001). The association between night blindness and vitamin A was made in 1925 by Frederica and Holm (Frederica, Holm, 1925), who recognized that vitamin A-deprived rats formed visual pigments at a slower rate than their normal-fed counterparts. Yudkin showed that vitamin A existed in the pig retina (Yudkin, 1931). However, it was Wald who, starting in 1935 and continuing into subsequent years, found that the visual pigments of the retina, called rhodopsin, consists of a protein, opsin, combined with "retinene" (later shown to be retinaldehyde). In the following years, Wald and colleagues elegantly described the basics of the visual cycle that regenerates light sensitivity of rhodopsin after a bleach (Wald, 1968). Research in the biochemical and molecular basis of the visual action of vitamin A led to the discovery of visual G protein-coupled receptors and culminated in the determination of their atomic structures (Palczewski et al., 2000).

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In the late 1920s, additional cellular processes were identified to be vitamin A-dependent (Wolbach, 1925). Researchers showed that this action is mainly connected with the acidic form of the vitamin, all-*trans*-retinoic acid (RA). Further research on RA has shown that the acidic form of the vitamin functions in diverse processes including cell differentiation and survival, immunity, reproduction, and embryonic development (Hall et al., 2011; Rhinn, Dolle, 2012; von Lintig et al., 2010). In the 1980s, it became clear that RA exerts these functions by modulating gene expression in various cell types of the body (Chytil, 1986; Lotan et al., 1980). In 1987, Chambon (Petkovich et al., 1987) and Evans (Giguere et al., 1987), and their respective co-workers, discovered the retinoic acid receptors (RARs). RARs are transcription factors of the nuclear hormone receptor gene family, which in conjunction with retinoid X receptors (RXRs), control gene transcription by binding to conserved DNA motifs (retinoic acid response elements) in promoter regions of about 500 target genes in the human genome (Balmer, Blomhoff, 2002). This type of action established RA as a hormone that is synthesized from dietary precursor molecules.

To support these functions, vitamin A precursors must be rendered available from the diet, transported, and metabolically converted into chromophore and RA hormone. Work by Olson (Olson, Hayaishi, 1965) and others delineated major steps in this metabolism. This was followed by identification of the importance of the serum transport protein for vitamin A by Goodman (Kanai et al., 1968) as well as intracellular retinoid-binding proteins by Chytil and Ong (Chytil, Ong, 1987). Norum and Blomhoff (Blomhoff et al., 1990) and many other researchers then established the basic facts of absorption and liver storage mechanisms for vitamin A. Vitamin A is the only vitamin that is stored in significant amounts in the body of vertebrates, thereby providing the key pro-survival advantage to endure periods with little to no dietary supplies of dietary precursor molecules.

Our laboratory entered this research field by studying insect vision and analyzing chromophore-deficient *Drosophila* mutants. Setting the stage for future research across diverse animal groups, we identified and characterized proteins for carotenoid absorption and bioconversion to chromophore (Kiefer et al., 2002; Oberhauser et al., 2008; von Lintig et al., 2001; von Lintig, Vogt, 2000). Subsequently, we showed that homologous proteins have similar functions in vertebrates, including humans (Amengual et al., 2011b; Amengual et al., 2013; Hessel et al., 2007; Kiefer et al., 2001; Voolstra et al., 2006). Our research also revealed that carotenoid absorption and bioconversion to vitamin A is regulated by demand and availability of dietary precursor molecules (Lobo et al., 2013; Lobo et al., 2010b). Comparative research in different animal classes provided insights as to how carotenoids and retinoids are transported and distributed throughout the body (Amengual et al., 2014a; Babino et al., 2015a; Isken et al., 2008; Widjaja-Adhi et al., 2015). This review summarizes and balances the advancements that have been made in the past two decades in our and others' laboratories with a particular focus on the role of carotenoids as chromophore precursor. We will follow the carotenoids' metabolic fate in the body from the gut to the eyes and consider the different biochemical strategies that animal classes have evolved to supply photoreceptors with chromophore.

2. Carotenoids

Carotenoids are a familiar sight as colorful pigments of fruits, flowers and vegetables. This class of isoprenoids comprises more than 1000 related compounds which contain up to 15 conjugated double bonds. The best known and eponymous representative of carotenoids is β-carotene (Figure 1). Other trivial names for carotenoids such as lycopene, lutein, zeaxanthin, and astaxanthin were in use long before the exact chemical structures of these compounds were determined. In hindsight, the monikers of carotenoids may seem undue but developed under functional analysis as opposed to stringent chemical elucidation. In 1974, The Nomenclature of Carotenoids was issued by the IUPAC with hundreds having been identified and even more looming. An online database searchable across several factors including chemical structures is maintained and updated with new discoveries (Yabuzaki, 2017).

Photosynthetic plants, protists and bacteria, as well as some heterotrophic bacteria and fungi can synthesize carotenoids. The initial steps of this pathway follow a common scheme that is like sterol and isoprenoid biosynthesis in most species (1,2). The first step exclusively devoted to carotenoid synthesis is the condensation of two molecules of geranylgeranyl pyrophosphate (C20) to phytoene (C40) catalyzed by phytoene synthase (Schledz et al., 1996). Synthesis of phytoene is an important branching point in plant isoprenoid metabolism and regulated by phytochrome and light during leaf development (von Lintig et al., 1997; Welsch et al., 2000; Welsch et al., 2003). The conversion of the colorless phytoene to the red colored lycopene occurs through four desaturation steps. In none-photosynthetic bacteria as well as in fungi, these steps are carried out by a single enzyme (Giuliano et al., 1986). In plants, this pathway employs several proteins that catalyze a sequence of desaturation and isomerization steps (Isaacson et al., 2004). This multistep pathway in higher plants is evidenced by mutant variants such as tangerine tomatoes which accumulate its tetra-*cis*-lycopene intermediate (Isaacson et al., 2002). Downstream in the biosynthetic pathway, all-*trans*-lycopene is the substrate for the introduction of ionone ring structures at both ends of the linear carbon chain of the carotenoid (Hugueney et al., 1995). These cyclization reactions yield β-carotene and α-carotene. The addition of functional groups is another notable strategy (Bouvier et al., 1998). Insertion of hydroxyl groups into the ionone rings creates lutein, the bright yellow pigment of daffodils and marigolds. Addition of keto-groups results in asthaxanthin, the vermilion color of flamingoes, salmon and crustaceans. The enormous diversity of carotenoids is achieved by modulation in length of the polyene chromophore and shifts of the conjugated double bonds that result in changes of the absorption spectra (Moise et al., 2014). Additionally, carotenoids can be chemically transformed to apocarotenoid metabolites through the introduction of oxygen at specific double bonds of their polyene backbone. The geometric forms all-(E)-form (all-*trans*) and (Z)-geometric isomers (*cis*) contribute to the chemically diversity of this class of natural compounds. Finally, chiral centres in cyclic end-groups also provide further multiplicity of optical isomers. Lutein, for example, contains three chiral centres at the 3, 3' and 6'-carbons with either R or S configurations. Zeaxanthin and meso-zeaxanthin, the other macular pigments of the human eyes, are limited to two at the 3 and 3'-carbons which exist in 3R,3R' configuration in zeaxanthin and 3R.3S' configuration in meso-zeaxanthin.

3. Carotenoid functions

Carotenoids are accessory pigments in the antennae of chloroplasts, where they augment the light-harvesting capacity by absorbing light in the blue-green range of the visible spectrum (450–550 nm) and transferring the energy to chlorophyll. They are also involved in photo-protection of the photosynthetic apparatus and other cellular structures (Demmig-Adams, Adams, 2002). The properties of carotenoids in photosynthetic systems continue to place a great deal of inquiry and inspire innovation in the field. For reviews based on this subject matter, carotenoid excited singlet states, we refer to (Hashimoto et al., 2018) and (Musser, Clark, 2019). Time-resolved spectroscopy of singlet fission in carotenoid aggregates even has revealed them as potential singlet fission sensitizers for solar panels (Billsten et al., 2005; Musser et al., 2015; Sineshchekov et al., 1972).

In daily life, carotenoids delight our senses as colors and expedite communication even over evolutionary borders. These colorful pigments attract pollinators such as birds and insects and advertise ripe fruits in exchange for seed dispersal. They maintain ranks in schools of fish and influence the mating choice of birds (Blount et al., 2003; Faivre et al., 2003). Feathers of some birds even contain crimson and burgundy colored carotenoids that are metabolically derived from dietary plant-produced carotenoid precursor molecules (Berg et al., 2013). Crustaceans such as lobster use carotenoids for blue coloration (Cianci et al., 2002). Once boiled the original reddish carotenoid color appears due to denaturing of the blueish protein-carotenoid complex.

Human blood and tissues also retain considerable amounts of carotenoids (Figure 2). In the eyes, the macular pigments have been chemically identified as the carotenoids lutein, zeaxanthin, and meso-zeaxanthin (Bernstein et al., 2016; Bone et al., 1985). Though distributed throughout the retina, these carotenoids are enriched in the fovea in primate retinas and confer its yellow colour. Hence, the fovea is traditionally known as the *macula lutea*, or 'yellow spot'. The macular pigments may protect the retina against light damage (Barker et al., 2011; Widjaja-Adhi et al., 2018) and reduce the adverse impact of light scattering and chromatic aberration, thereby optimizing contrast sensitivity of the retina (Hammond et al., 2013). These properties have led to the hypothesis that macula pigments may protect against the development of age related macular degeneration (AMD) (Bernstein et al., 2016; Mares, 2016). Epidemiological studies support this role and revealed that individuals with lower concentrations of serum carotenoids and macular pigment optical density measurements are at a higher risk of developing AMD. Conversely, nutritional supplementation and diets rich in lutein and zeaxanthin readily impact MP concentrations and reduce the risk of progression to advanced AMD (Chew et al., 2014). The light filtering properties of carotenoids also can provide modest protection against ultraviolet (UV)-induced erythema in the skin (Kopcke, Krutmann, 2008).

Several epidemiological studies have reported that plasma carotenoid levels are inversely correlated with the incidence of cardiovascular diseases (Bohn et al., 2017). This association has been reported for total plasma carotenoids and individual carotenoids such as lycopene, β-carotene, and lutein (Leermakers et al., 2016). There are several potential mechanisms through which carotenoid consumption may affect the etiology of these diseases. The most

commonly cited is through the antioxidant action of carotenoids. The antioxidant properties of carotenoids may decrease lipid peroxidation and eventually reduce oxidative stress and inflammation responses in human cells and tissues (Edge, Truscott, 2018). Additionally, local production of signaling molecules including RA from carotenoids may influence the activity of nuclear receptors (Alvarez et al., 2014).

Higher blood levels of carotenoids have been also associated with prevention of various forms of cancer, particularly prostate cancer. The effects may involve antioxidant properties, interactions with signaling molecules such as NFκB (Linnewiel-Hermoni et al., 2014), and interactions with the metabolism and transport of other lipids, particularly cholesterol (Palozza et al., 2012). Notably, also adverse effects of certain carotenoids, β-carotene, on cancer have been reported (Omenn et al., 1996; Wang, Russell, 1999). Studies revealed that levels, environmental factors, and genetics may play a role for this process. However, many molecular details of carotenoid action remain undefined and more research is required to analyze the distribution, metabolism, and function of carotenoids in relation to different forms of cancer.

Living organisms chemically transform carotenoids by oxidative cleavage at specific alkene bonds to generate a unique series of apocarotenoid metabolites (Figure 3). In all kingdoms of life, apocarotenoids serve as chromophore of photopigments. The occurrence of retinaldehyde in bacteriorhodopsin qualifies this lipid as evolutionarily among the oldest photochemical compounds (Jung, 2007). Similarly, most animals metabolize carotenoids to chromophore. The chromophore function of retinoids is clearly defined by the compounds' chemical and physical properties. The interaction of delocalized π-electron system with electromagnetism underlies the light harvesting and light filtering properties of these compounds.

Cleavage of carotenoids also yields β-ionone, α-ionone, bixin (annatto), saffron, and norisoprenoids. These apocarotenoids are of significant economic value as food colorants, cosmetic (Bouvier et al., 2003a; Bouvier et al., 2003b), and aromatic compounds (Mendes-Pinto, 2009).

Apocarotenoids also are used as hormones which upon receptor activation regulate various physiological processes. In plants, abscisic acid and strigolactones influence processes, as diverse as seed dormancy, morphogenesis and environmental adaptation (Al-Babili, Bouwmeester, 2015; Schwartz et al., 1997). More recently, β-cyclo-citral and zaxinone have been identified as growth factors in plants (Dickinson et al., 2019; Wang et al., 2019). In vertebrates, RA regulates gene expression throughout the life cycle and is responsible for most, if not all, non-visual physiological effects of the vitamin. Interestingly, some cyanobacteria can also produce RA and its hydroxylated derivatives from carotenoids (Alder et al., 2009). Cyanobacteria blooms may result in aquatic concentrations of this hormone that can harm amphibians in eutrophic habitats (Wu et al., 2012). Animals also transform carotenoids into long chain apocarotenoids (>C20). Birds use 3-hydroxy-dehydro-β–10'-apocarotenol (galloxanthin) in oil droplets of the retina to modulate spectral sensitivity of visual pigments in adjacent cone photoreceptors (Toomey et al., 2016). In mammals, emerging evidence suggests these compounds are biologically active modulators of

physiological processes (Aydemir et al., 2016; Harrison, Quadro, 2018; Lian et al., 2007; Narayanasamy et al., 2017). For instance, it has been reported that pharmacological doses of apo-10'-lycopenoic acid can protect against liver damage (Ip et al., 2013). Furthermore, β −13-apo-carotenone and certain lycopenoids act as RAR antagonists (Eroglu et al., 2012; Narayanasamy et al., 2017). A recent study indicate that apocarotenoids different than RA may act as signal molecules on their own right in physiological processes of mammals (Costabile et al., 2016).

4.   The enzymatic conversion of carotenoids into apocarotenoids

4.1.   The discovery of carotenoid cleavage dioxygenases

Oxidative cleavage of carotenoids at a specific position of the polyene chain had been proposed as the method for apocarotenoid production in all living organisms. However, the identity and molecular nature of the involved enzymes remained elusive for a long time. By analyzing the genetic basis of the abscisic acid-deficient corn mutant, *vp14* (*viviparous* 14), the first gene encoding a carotenoid cleavage dioxygenase (CCD) was identified and the encoded enzyme biochemically characterized (Schwartz et al., 1997). Recombinant VP14 catalyzes oxidative cleavage at position C10, C11 of 9-*cis*-violaxanthin to yield xanthoxin.. Xanthoxin is then further metabolized to abscisic acid. The researchers proposed that related CCDs catalyze oxidative cleavage of carotenoids in other organisms. We succeeded in the identification of three distinct types of metazoan CCDs encoded by the *ninaB*, *BCO1*, and *BCO2* genes (Kiefer et al., 2001; von Lintig, Vogt, 2000). Simultaneously, a classical protein purification and sequencing approach conducted by Adrian Wyss identified the BCO1 gene from chicken (Wyss et al., 2000). We used a cloning and expression strategy to biochemically characterize these enzymes which employed carotenoid producing *E. coli* strains (Figure 4). These *E. coli* strains lose their bright yellow or red colors and turn pale when a CCD is expressed that splits the pigments into apocarotenoids. The discoloration occurs because the blue-shifted spectrum and lower extinction coefficient of the apocarotenoid products. This robust and reliable test system was instrumental in overcoming initial difficulties with the heterologous expression and biochemical characterization of these enzymes. These enzymes catalyze a reaction at the lipid aqueous interface and can form insoluble misfolded aggregates when expressed in *E. coli*. The challenging biochemistry of CCDs had led to many conflicting results about this enzyme class. For instance, the retina pigment epithelium protein of 65 kDa (RPE65), the first molecularly identified metazoan member of the CCD family (Hamel et al., 1993), was proposed to be a retinoid-binding protein rather than an enzyme catalyst (Gollapalli et al., 2003; Mata et al., 2004). Moreover, the existence of a vitamin A forming enzyme was questioned by some researchers because of the failure to measure its activity in cell free extracts (Hansen, Maret, 1988). Over the years, advanced protocols for the heterologous expression and biochemical characterization of CCDs were established and enzymatic properties of CCDs were scrutinized. Amongst others, these analyses revealed an additional enzymatic property of this enzyme class that is critical for their visual function. Some CCDs possess intrinsic isomerase activity and catalyze geometric isomerization of double bonds within the polyene backbone of carotenoids. The CCD, encoded by the insect neither inactivation nor after potential gene B (*ninaB*) gene (von Lintig et al., 2001), catalyzes a combined oxidative cleavage at position

C15,C15' and isomerization at position 11',12' of its carotenoid substrates to directly yield visual chromophore (Babino et al., 2016; Oberhauser et al., 2008) (Figure 5). In vertebrates, oxidative cleavage of the carotenoid and geometric isomerization of the resulting retinoid are catalyzed by two distinct family members, respectively encoded by the *BCO1* and *RPE65* genes (Figure 6). BCO1 encodes a β-carotene-15,15'-dioxygenase that splits β-carotene into two molecules of retinaldehyde (Wyss et al., 2000). RPE65 acts as a retinoid isomerase and catalyst of the conversion of retinyl esters (RE) to 11-*cis*-retinol (Jin et al., 2005; Moiseyev et al., 2005; Redmond et al., 2005a). The third vertebrate CCD is encoded by the *BCO2* gene and catalyzes cleavage at position C9,C10 and C9',C10' of carotenoids (Kiefer et al., 2001). Though originally characterized as β-carotene metabolizing enzyme, BCO2 is promiscuous and displays broad substrate specificity for various carotenoids and apocarotenoids, including lycopene, zeaxanthin, lutein, and canthaxanthin (Amengual et al., 2011b; Dela Sena et al., 2016; Hu et al., 2006; Kelly et al., 2018; Lobo et al., 2012; Mein et al., 2010). One study indicates that human BCO2 is enzymatically inactive and that this inactivity favors the accumulation of macula pigment in the retina (Li et al., 2014). However, other studies provided evidence that human BCO2 catalyzes carotenoid breakdown when expressed in *E. coli* cells (Palczewski et al., 2014; Poliakov et al., 2017). Additionally, BCO2 cloned from macaques, a primate with macula pigments, converts zeaxanthin into apocarotenoids (Babino et al., 2015a), indicating that ocular accumulation of carotenoids is not dependent on an inactive BCO2 enzyme.

4.2.   Physiological roles of BCO1 and BCO2 in mammals

All three CCDs display distinct subcellular localizations. BCO1 exists in the cytoplasm, RPE65 at the endoplasmic reticulum, and BCO2 resides at the inner mitochondrial membrane (Golczak et al., 2010; Kowatz et al., 2013; Palczewski et al., 2014). On tissue and organ levels, BCO1 and BCO2 are expressed in many human tissues and cell types (Lindqvist, Andersson, 2002; Lindqvist et al., 2005), whereas RPE65 expression is restricted to the mammalian RPE that nourishes and maintains the adjacent photoreceptor in the retina with chromophore (Hamel et al., 1993). The expression pattern of BCO1 and BCO2 indicates that most cell types and tissues can metabolize carotenoids to apocarotenoids and can synthesize vitamin A. In enterocytes of the intestine, BCO1 expression is controlled by vitamin status via the transcription factor ISX (Lobo et al., 2013). In other tissues, BCO1 expression is controlled by peroxisome proliferator activator receptors (Boulanger et al., 2003; Lobo et al., 2010a). Knowledge about the transcriptional control of the *BCO2* gene is limited. A recent study showed that *BCO2* mRNA is highly expressed in the peripheral retina and at low level in the central (macula) retina of the human eye. This expression pattern may contribute to the specific carotenoid distribution in the human retina (Voigt et al., 2019). Differential expression of BCO2 in tissues has also been associated with skin coloration in birds (Eriksson et al., 2008) and more recently in lizards (Andrade et al., 2019). In human cell lines, BCO2 expression is induced by oxidative stress (Babino et al., 2015a). The latter regulation and the broad substrate specificity of BCO2 indicate that the enzyme acts as a carotenoid scavenger and may explain why carotenoid levels decrease in chronic disease states that are associated with inflammation and oxidative stress.

The generation of mouse models deficient for BCO1 and BCO2 set the stage for an in-depth analysis of the metabolism of dietary carotenoids (Amengual et al., 2011b; Hessel et al., 2007). These studies identified BCO1 as major β-carotene metabolizing enzyme and key enzyme for vitamin A production (Amengual et al., 2013). In its absence, only trace amounts of β-apo-10'-carotenols are produced from supplemented β-carotene by BCO2 (Amengual et al., 2011a; Amengual et al., 2013). Similarly, humans with inherited BCO1 deficiency show elevated β-carotene and reduced vitamin A levels (Lindqvist et al., 2007). Consistent with BCO2's enzymatic properties and subcellular localization, xanthophylls accumulate in the mitochondria of BCO2-deficient mice (Amengual et al., 2011b). This accumulation of xanthophyll increases oxidative stress in tissues and reduces mitochondrial respiration rates (Amengual et al., 2011b; Lobo et al., 2012; Palczewski et al., 2016). Xanthophylls accumulate in oxidized form in mouse tissues (Amengual et al., 2011b; Palczewski et al., 2016). 4′,5′-Didehydro-retro-β-carotene-3,3′-dione (rhodoxanthin) and (6RS,6RS)-ε,ε-Carotene-3,3'-di-one were respectively identified as zeaxanthin oxidation products in white adipose tissues (Widjaja-Adhi et al., 2015) and in blood and liver (Amengual et al., 2011b; Palczewski et al., 2016). Lower levels of oxidized carotenoids were measured in lung and heart of CCD deficient mice (Amengual et al., 2011b; Amengual et al., 2013; Hessel et al., 2007). Xanthophyll also accumulate in the eyes of CCD knockout mice with higher levels in the RPE than in the neuronal retina (Li et al., 2014; Widjaja-Adhi et al., 2015). However, levels of carotenoids in the eyes were low when compared to liver, fat, and blood (Babino et al., 2015a). This observation clearly indicates that mice lack mechanisms for ocular carotenoid accumulation. BCO2-deficient mice also accumulate lycopene in different tissues, whereas levels of supplemented lycopene were indistinguishable between wild type control and BCO1-deficient mice (Ford et al., 2010).

Naturally occurring mutations in the *BCO2* gene have been associated with the yellow skin phenotype in chicken (Eriksson et al., 2008) and the yellow fat phenotype in cattle and sheep (Berry et al., 2009; Tian et al., 2009; Vage, Boman, 2010). These findings demonstrate that turnover of carotenoids by CCDs plays an important role in the control of carotenoid homeostasis (Figure 7). Given the importance of carotenoids in vision, development, reproduction, and immunity, it has been speculated that *BCO2* mutations in domestic animals help to adapt them to artificial diets low in carotenoids that are vastly different from those of their wild-living ancestors (Fallahshahroudi et al., 2019).

4.3. CCD structure and chromophore production

The structures of an apocarotenoid-oxygenase (ACO) (Kloer et al., 2005; Sui et al., 2014), the plant enzyme VP14 (Messing et al., 2010), and bovine RPE65 (Kiser et al., 2012; Kiser et al., 2009) have been resolved at atomic resolution. These analyses revealed that the overall architecture of CCDs is well conserved. The basic structural fold is a rigid seven-bladed β-propeller covered by a half-dome (Figure 8A). The Fe (II) of the active center is accessible through long non-polar tunnels. The ferrous iron center in CCDs is invariably coordinated by four His residues, with three Glu residues forming a second coordination sphere. Moderate resolution of the crystal structures of ACO and RPE65 show average Fe-N^e bond lengths of ~2.1–2.2 Å, a distance consistent with the 2.15 Å Fe-N^e bond length measured for RPE65 by X-ray absorption spectroscopy (XAS) (Khadka et al., 2019; Kiser et al., 2012).

As indicated in the structure of iron-free ACO, this platform for iron-binding is rigid and does not change upon iron-binding (Kloer et al., 2005). Mutagenesis studies of the key metal-binding first and second sphere His and Glu residues indicate that iron is absolutely required for CCDs to perform their catalytic roles and that both first and second sphere ligands contribute to iron-binding and catalytic function (Poliakov et al., 2005; Redmond et al., 2005b; Sui et al., 2017; Sui et al., 2016; Takahashi et al., 2005). The requirement for a divalent iron in CCD catalysis is well documented by several studies (Kiefer et al., 2001; Lindqvist, Andersson, 2002; Moiseyev et al., 2006; Redmond et al., 2001; Schwartz et al., 1997). The putative role of Fe(II) in canonical double bond cleaving CCDs such as BCO1, ACO and VP14 is to activate oxygen for cleavage of carotenoid/apocarotenoid substrates (Borowski et al., 2008; Kloer, Schulz, 2006). It is now generally accepted that the incorporation of dioxygen follows a dioxygenase reaction mechanism (Babino et al., 2016; Dela Sena et al., 2014; Schmidt et al., 2006; Sui et al., 2015). In the RPE65 catalyzed isomerization reaction, Fe(II) acts as a Lewis acid that polarizes the ester group and is not involved in the activation of molecular oxygen (Kiser et al., 2015). For a detailed review of the structural arrangement of the active center and the mode of enzyme catalysis of CCDs we here refer to an excellent review article (Daruwalla, Kiser, 2019).

Native bovine RPE65 has been crystallized in complex with the retinoid-mimetic drug ACU-4429 (emixustat) (Kiser et al., 2015). This study revealed that the active retinoid-binding cavity is located near the membrane-interacting surface as well as a Fe-bound palmitate ligand positioned in an adjacent pocket. The structural arrangement of the enzyme-substrate complex (Michaelis complex) of a canonical double bond cleaving CCD has not yet been determined. Our efforts to yield structures of recombinant BCO1 and BCO2 were met with a major difficulty. The greatest obstacles were co-purification of the bacterial chaperone GroEL and a lack of a native tissue source from which the enzymes can be purified in enough amounts. For recombinant ACO a crooked, rod-shaped electron density feature observed in the active site cavity after soaking ACO crystals with substrate was attributed to a bound apocarotenoid (Kloer et al., 2005). However, electron densities for both the β-ionone ring and alcohol tail of the apocarotenoid were invisible and only the central part of the substrate density was observed. These crystals were obtained in the presence of polyethylene glycol and the detergent octylpolyoxyethylene, molecules that also bear elongated carbon chains resembling an isoprene chain. The possibility that the observed electron density is a detergent was recently confirmed by a refined structural analysis of ACO (Sui et al., 2014). Improved protocols for the expression of CCDs as well as methods for the generation of metal substituted CCDs provides novel tools to analyze the interaction between CCDs and their carotenoid substrates (Sui et al., 2018).

The current knowledge allows us to hypothesize that the architecture of the substrate tunnels of CCDs significantly contributes to the selectivity of chromophore production. The overwhelming majority of animals use a single chromophore to avoid ambiguity in spectral sensitivity of visual pigments: Vertebrates use retinal (vitamin A_1) and 3-dehydroretinal (vitamin A_2), arthropods use retinal and 3-hydroxy-retinal (vitamin A_3), and some crustaceans use 4-hydroxy-retinal (vitamin A_4) (Goldsmith, 2013). For vitamin A_1 production in mammals, BCO1 selectively interacts with the non-substituted β-ionone ring of provitamin A carotenoids (Kelly et al., 2018; Lindqvist, Andersson, 2002) (Figure 8B).

Xanthophylls such as zeaxanthin and lutein with two hydroxylated β-ionone ring sites are not a substrate for the recombinant BCO1 enzyme (Kelly et al., 2018). Though the structure of BCO1 has not been solved, it can be assumed that the architecture of the enzyme hinders substituted ionone rings from entering the substrate tunnel. When comparing the predicted structures of BCO1 and BCO2, we reasoned that the diameter of the entrance of the substrate tunnel may be critical for substrate selectivity. The opening of the substrate tunnel should be wider in BCO2 than in BCO1 because BCO2 can accommodate bulky hydroxylated ionone rings in the substrate tunnel (Figure 8C). Comparison of the predicted structures of BCO1 and BCO2 identified two candidate amino acids (Trp270 and Leu168) that narrow the mouth of the substrate tunnel in BCO1. Exchange of these amino acids with the corresponding amino acids of BCO2 (Trp270Phe and Leu168Gly) by site-directed mutagenesis produced a mutant BCO1 enzyme that was able to cleave zeaxanthin with two hydroxylated ionone rings (Kelly et al., 2018). Thus, a small change in an overall conserved fold provides the structural basis of the ring site selectivity of BCO1 and BCO2.

A directional interaction of CCDs with ionone rings of the carotenoid substrates also plays a role in substrate recognition of insect NinaB (Oberhauser et al., 2008). Studies with recombinant NinaB from the wax moth Galleria showed that the enzyme only uses carotenoids with two ionone rings as substrates (Babino et al., 2016). In this respect, NinaB differs from mammalian CCDs which can convert apocarotenoids with one ionone ring site (Kelly et al., 2018) as well as the acyclic lycopene (dela Sena et al., 2013; Kiefer et al., 2001). Additionally, moth NinaB prefers 3-hydroxy-β-ionone ring sites over non-substituted ring sites to synthesize 3-hydroxy-retinal (Oberhauser et al., 2008), the unique vitamin A_3 chromophore of higher flies and butterflies (Seki, 1998; Vogt, 1983). Mammalian BCO2 also preferentially interact with hydroxylated ionone rings of carotenoids (Kelly et al., 2018; Mein et al., 2010). This enzymatic property of BCO2 plays an important role for quality control of chromophore production in mammals (Fig. 8). BCO2 removes the non-canonical ring sites from asymmetric provitamin A carotenoids such as β-cryptoxanthin (Kelly et al., 2018). The resulting β–10'-apocarotenal is subsequently converted by BCO1 into vitamin A_1-aldehyde. In the mouse intestine, all metabolites of this pathway were identified upon β-cryptoxanthin supplementation (Kelly et al., 2018).

Specific modification of the β-ionone ring by cytochrome P450 family member is another notable concept for chromophore synthesis. In lower vertebrates, the cytochrome P450 family member, CYP27c1, converts retinal (vitamin A_1) into 3,4-didehydroretinal (vitamin A_2). Genetic disruption of *cyp27c1* abrogates the zebrafish' ability to red-shift its photoreceptor spectral sensitivity (Enright et al., 2015). A cytochrome P450 enzyme also has been implicated in the conversion of retinaldehyde (vitamin A1) into 3-hydroxy-retinal (vitamin A3) in flies (Seki et al., 1998). *Drosophila* can also convert supplemented β-carotene to zeaxanthin which then is converted by NinaB into vitamin A_3 (Voolstra et al., 2010). Though not directly related to chromophore production, the cytochrome P450 enzyme CYP2J19, acts as a carotenoid ketolase in astaxanthin synthesis in birds (Lopes et al., 2016). Thus, stereo- and region-selectivity of CCDs as well modifications of the β-ionone ring play critical roles for chromophore production in different animal species.

5. The Absorption of Carotenoids

5.1. Analysis of Drosophila ninaD mutant revealed protein-dependency of fat soluble vitamin absorption

In contrast to carotenogenic organisms, animals must acquire carotenoids from the diet. For many years it was assumed that the uptake of dietary lipids, including carotenoids, occurs via passive diffusion. However, studies in cell culture revealed that carotenoid but not retinoid absorption is saturable (During et al., 2002) and it is now well accepted that it depends on scavenger receptors (Reboul, Borel, 2011). To our knowledge, the protein-dependency of this process was firstly demonstrated by our analysis of the *Drosophila* mutant *ninaD* (Kiefer et al., 2002) (Figure 9). Initially, this mutant was identified in a large screen for visual pigment-deficient mutants and was shown to lack chromophore when raised on standard corn meal chow (Giovannucci, Stephenson, 1999; Stephenson et al., 1983). We searched the genomic region to which the *ninaD* gene was mapped and focused on a gene that encodes a class B scavenger receptor. Class B scavenger receptors were previously characterized to facilitate cholesterol transport between high density lipoproteins and cells in mammals (Acton et al., 1996; Kozarsky et al., 1997). We reasoned that NinaD may facilitate carotenoid uptake in the fly because of the similar chemical characteristics between carotenoids and cholesterol. Our candidate approach identified a nonsense mutation in the *ninaD* gene that led to a premature stop codon. We also showed that expression of a wild type *ninaD* transgene rescued the blind phenotype and restored visual pigment synthesis in the mutant (Kiefer et al., 2002). Later, it was shown that *ninaD* mutant flies also lack tocopherols (vitamin E) and that *ninaD* is expressed in the midgut of the larva (Voolstra et al., 2006; Wang et al., 2007). This expression pattern coincides with the time point when the larva acquires carotenoids for chromophore production during compound eye development in the pupal stage of this holometabolous insect. When expressed in *Drosophila* Schneider cells, NinaD localizes to the plasma membrane and facilitates carotenoid uptake from synthetically mixed micelles (Voolstra et al., 2006) (Figure. 9 B, C). A second NinaD homologous scavenger receptor encoded by the *santa maria* gene facilitates carotenoid uptake into neuronal and glial cells where carotenoids are converted to chromophore by NinaB (Wang et al., 2007). The transport of chromophore to photoreceptors employs a CRAL-TRIO domain protein encoded by the *pinta* gene (Wang, Montell, 2005). Eyes of the p*inta* mutant fly strain contain chromophore but display highly reduced levels of rhodopsin (Voolstra et al., 2010). This finding indicates that Pinta is required for the transport of chromophore from its place of production to photoreceptor cells. The dependency of retinoid transport from Pinta is demonstrated by another study. Ectopic expression of a *ninaB* transgene renders the fly eyes, carotenoid and chromophore deficient (Voolstra et al., 2010). Retinoids only can be used as chromophore precursors in the fly when supplied in very high doses with the chow (Wang et al., 2010) or when an alcoholic retinoid solution is directly applied on the eyes with a brush(Voolstra et al., 2010).

Research in other insect species confirmed the initial findings about the *ninaD* gene and carotenoid transport. Analysis in the silk worm *Bombyx mori* showed that carotenoid absorption is mediated by a NinaD-related protein encoded by the *Yellow Cocoon* gene (Sakudoh et al., 2010). Additionally, a carotenoid-binding protein (CBP) is encoded by the

Yellow Blood gene. CBP is a member of the family of steroidogenic acute regulatory (StAR) proteins and required for carotenoid accumulation in the silk gland (Sakudoh et al., 2010).

5.2. Class B scavenger receptors in vertebrate fat-soluble vitamin absorption

In mammalian cell lines, the scavenger receptor class B type 1 (SR-B1) (During et al., 2005; During et al., 2008; Goncalves et al., 2014; Reboul et al., 2006; van Bennekum et al., 2005; Voolstra et al., 2006; Widjaja-Adhi et al., 2015) and cluster determinant 36 (CD36) facilitate carotenoid uptake from micelles (During et al., 2005; Reboul et al., 2005). The involvement of SR-B1 in the intestinal absorption of β-carotene and zeaxanthin was later confirmed in mice (van Bennekum et al., 2005; Widjaja-Adhi et al., 2015). Studies in mice also demonstrated that SR-B1 facilitates the intestinal absorption of fat soluble vitamins E and K (Goncalves et al., 2014; Reboul et al., 2006). Moreover, it was demonstrated that CD36 also contributes to the intestinal absorption of dietary isoprenoids in mice (Borel et al., 2013; Shyam et al., 2017b). The critical role of SR-B1 in this process is beautifully demonstrated by the white recessive mutation of canary birds. This phenotype is caused by a splice site mutation in the SR-B1 gene (Toomey et al., 2017). The mutant bird shows white feather coloration and very low levels of carotenoids in blood and tissues. These birds also suffer from severe vitamin A deficiency and depend on supplementation with preformed vitamin A via the diet (Preuss et al., 2007).

Scavenger receptors are glycosylated transmembrane proteins with a large extracellular domain. Structural prediction, based on the crystal structure of the CD36 family member lysosomal membrane protein 2 (LIMP II), (Neculai et al., 2013) indicate the presence of a large cavity traversing the entire length of the protein that serves as a tunnel for lipid transfer from extracellular to cellular compartments (Rodrigueza et al., 1999; Yu et al., 2012). Mammalian CD36 and SRB1 have been associated with a wide range of physiological processes. Their broad substrate specificity relies on their ability to recognize similar molecular patterns rather than specific epitopes. CD36 substrates include carotenoids, long chain fatty, native or modified lipoproteins, thrombospondin-1, collagen, apoptotic cells, amyloid B, and malaria-infected erythrocytes (Febbraio, Silverstein, 2007). CD36 is expressed in muscle, adipose tissue, intestine and the capillary endothelium, where it facilitates long chain fatty acid uptake into target cells in capillary beds of tissues (Abumrad et al., 1993; Greenwalt et al., 1992), (Goldberg et al., 2009). CD36-deficient mice display among other defects, significantly increased levels of circulating fatty acids (Cifarelli, Abumrad, 2018). SR-B1 binds high-density lipoproteins (HDLs) (Connelly et al., 1999) and facilitates selective cellular uptake of cholesterol in mammalian steroidogenic tissues (Acton et al., 1996). SR-B1 is expressed in the liver, intestine, macrophages, adrenal gland, and ovary (Acton et al., 1994; Cifarelli, Abumrad, 2018). SR-B1-deficient mice develop hypercholesterolemia and multiple pathologies, including male sterility (Acton et al., 1996).

5.3. Regulation of carotenoid absorption and vitamin A production in the intestine

Studies in human and experimental animals provided evidence that the vitamin A status of the subject affects carotenoid absorption (Borel et al., 2011; Meyers et al., 2014) and bioconversion to vitamin A in the intestine (Lala, Reddy, 1970; van Vliet et al., 1996; Villard, Bates, 1986). It is now generally acknowledged that vitamin A status affects β-

carotene absorption and bioconversion efficiency via a negative feedback loop (Bachmann et al., 2002; Borel et al., 2015). The control of vitamin A production in intestinal enterocytes is a rare example of negative transcriptional feedback regulation. The *Bco1* gene is expressed by default when there is a demand for vitamin A (Bachmann et al., 2002; Seino et al., 2008). However, if preformed vitamin A or β-carotene is present in the diet, enterocytes rapidly cease production of vitamin A (Bachmann et al., 2002; Lobo et al., 2010b; Seino et al., 2008). Similarly, when the body's vitamin A stores are filled, the gene expression of *Bco1* is repressed in brush border cells (Lobo et al., 2010b). This regulation avoids excess vitamin A production that can be detrimental (Lobo et al., 2013; Widjaja-Adhi et al., 2017).

The repressor protein of vitamin A production has been identified as the intestine specific homeobox transcription factor ISX (Choi et al., 2006; Seino et al., 2008). This transcription factor binds to conserved motifs in promoter region of the *Bco1* gene (Lobo et al., 2013; Widjaja-Adhi et al., 2015) (Figure 10). The small molecule inducer that regulates the activity of the repressor protein is retinoic acid (Lobo et al., 2010b). The acidic form of the vitamin binds to retinoic acid receptors to induce *Isx* mRNA expression in the intestine (Lobo et al., 2010b). In turn, ISX binds to the promoters of the *Bco1* and *SR-B1* gene and represses their mRNA expression (Lobo et al., 2013; Widjaja-Adhi et al., 2015).Thus, in the presence of elevated levels of RA protein levels of SR-B1 and BCO1 decrease in the enterocytes and absorption and bioconversion of carotenoids are diminished. This indirect transcriptional regulation of the repressor protein is required because the *Bco1* gene promoter is controlled by vitamin A-independent mechanisms in other cell types of the body (Boulanger et al., 2003; Lobo et al., 2010b). In the intestine of vitamin A sufficient mice, ISX is expressed at higher levels in distal rather than in proximal parts (Seino et al., 2008). The ISX target genes, encoding BCO1 and SRB1, reveal a reverse pattern of expression (Seino et al., 2008) which guarantees that provitamin A carotenoids are effectively acquired from the diet in the vitamin A deficient state. In a vitamin A sufficient scenario, the shutdown of vitamin A production prevents excessive accumulation of retinoids and imbalances in intestinal RA production (Widjaja-Adhi et al., 2017).

Significant SNPs were found in the genes encoding *BCO1*, *SR-B1*, and the transcription factor ISX (Borel et al., 2011; Borel et al., 2015; Ferrucci et al., 2009; Leung et al., 2008). Interestingly, SNPs in *BCO1* are associated with macular pigment density (Meyers et al., 2013). This finding is surprising at first glance since xanthophylls are not a substrate for the vitamin A forming enzymes. However, the vitamin A-dependent regulation of *SR-B1* expression in the intestine explains the interaction between xanthophyll accumulation and β-carotene metabolism. It has long been known in chicken that absorption of xanthophyll is reduced in the presence of dietary preformed vitamin A (Thompson, 1975).

Genetic makeup also influences the conversion rate of provitamin A carotenoids (Grune et al., 2010; Leung et al., 2008; Lietz et al., 2012) and may constitute the basis for the low and high responder phenotypes described in the general European male population (Borel et al., 1998). Additionally, several SNPs in proteins that affect lipid and lipoprotein metabolism have been associated with carotenoid blood levels (Borel, Desmarchelier, 2018). The latter association indicates significant interactions between carotenoid and lipid metabolism. Such interactions have been demonstrated in mouse models (Palczewski et al., 2016). Most of the

genetic data in humans stem from the analyses of β-carotene, lycopene, and lutein fasting blood levels, but it is assumed that these genetic variations also modulate the bioavailability of the other carotenoids found in human blood and tissue (Borel, Desmarchelier, 2018).

6. Enzyme Classes Involved in Retinoid Metabolism

The term retinoid was coined by Sporn and colleagues in the mid-1970s (Sporn et al., 1976). Retinoids comprise both natural and synthetic compounds with structural resemblance to all-*trans*-retinol, with or without the biological activity of vitamin A. Hence, vitamin A (by definition all-*trans*-retinol) is a natural retinoid. The metabolism of retinoids has been recently reviewed in several excellent articles (Blaner et al., 2016; Chelstowska et al., 2016; D'Ambrosio et al., 2011; Kiser et al., 2014). For completeness, we will introduce the enzyme classes of these metabolic processes because they will later become important for the discussion of ocular retinoid metabolism (Figure 11). Retinaldehyde, the product of β-carotene splitting, can be either oxidized to all-*trans*-RA or reduced to all-*trans*-retinol. All-*trans*-retinol is converted to retinyl esters (RE). The alcohol and ester form of vitamin A are the predominant retinoids in tissues, whereas the acidic form of the vitamin exists only in very low (nanomolar) concentrations in most tissues (Kane et al., 2010). The tissue concentrations levels of RA are tightly controlled, and even small amounts of this hormone-like compound are sufficient to elicit profound cellular responses through the activation of RA receptors (Giguere et al., 1987; Petkovich et al., 1987). RARs belong to the superfamily of nuclear receptors. Nuclear receptors function as ligand-activated transcription factors. They were first recognized as the mediators of steroid hormone signaling and provided an important link between transcriptional regulation and physiology. The human genome encodes 48 members of this transcription factor family, including classic endocrine receptors that mediate the actions of steroid hormones, thyroid hormones, and the fat-soluble vitamins A and D, but also nuclear receptors that acts as lipid sensors and in many other cellular processes (Chawla et al., 2001). Nuclear receptors contain two major domains: A amino-terminal DNA binding domain with two conserved zinc finger motifs that is connected with a flexible hinge region to the carboxy-terminal ligand binding and dimerization domain. Upon ligand binding, nuclear receptors undergo a conformational change that result in the dissociation of corepressors and in the recruitment of coactivator proteins to enable transcriptional activation. RARs form obligate dimers with retinoid X receptors (RXRs) and control transcription by binding to conserved DNA motifs (retinoic acid response elements) in promoter regions of about 500 target genes in the human genome (Balmer, Blomhoff, 2002). The amount of RA in tissues is tightly controlled throughout the mammalian life cycle by cytochrome P450-dependent hydroxylases CYP26A1, CYP26B1 and CYP26C1 that catalyze the production of 4-hydroxy and 4-oxo-RA (Abu-Abed et al., 2001; Abu-Abed et al., 1998; Niederreither et al., 2002; Zhong et al., 2019). The responsiveness of CYP26A1 expression to RA indicates that this enzyme is a major contributor to the catabolism of acidic retinoids. This metabolic regulation is found for many CYP enzymes which catabolize dietary lipids and prevent their excessive accumulation. Regulation activities of the other RA catabolizing enzymes, CYP26B1 and CYP26C1, are more complex and description of their physiological roles are subject of ongoing research.

The interconversion of retinol to retinal is catalyzed by cytosolic alcohol dehydrogenases (ADHs) and microsomal retinol dehydrogenases (RDH) (Kedishvili, 2013; Napoli, 2012). The latter belong to the short chain dehydrogenase/reductase protein family (SDR). Adenine dinucleotide cofactors NAD(H) and NADP(H) are the redox carriers of these reactions. The enzymes bind their cofactors by a conserved sequence motif, the Rossmann-fold, which consists of six to seven parallel β-strands flanked by three to four α-helices (Hofmann et al., 2016; Kiser et al., 2014). ADHs and RDHs use different catalytic mechanisms with either a zinc atom or a tyrosine in the active center, respectively. ADHs and RDHs catalyze an equilibrium reaction which depends on the oxidative state of their redox carriers. Under physiological conditions, the ratio of NAD/NADH is around 1000 in the cytoplasm, and the ratio of NADP/NADPH is 0.005. Thus, enzymes using NAD as redox carriers catalyze the oxidation while enzymes using NADP catalyze reduction of retinoids.

Though ADHs and RDHs can use retinol and retinal as substrates, most of these enzymes can metabolize other alcohols including sterols (Kedishvili, 2013). Therefore, knowledge about the physiological functions of these enzymes stem from loss-of-function studies in knockout mice. (Kedishvili, 2013; Kumar et al., 2012; Napoli, 2012). The observed consequences of the gene knockouts are discussed below. In extraocular retinoid metabolism, RDH10 plays a critical role in RA synthesis (Sandell et al., 2007). DHRS3 has been identified as critical retinal reductase in the embryo (Billings et al., 2013) and post-developmentally in adult tissues (Adams et al., 2014; Zolfaghari et al., 2012). RDH1 contributes to retinoid homeostasis in adult tissues as well (Napoli, 2012; Obrochta et al., 2015; Zhang et al., 2007). RDH1-deficient mice display increased hepatic retinyl ester stores and altered body fat mass (Zhang et al., 2007). RDH11 is required for the maintenance of all-*trans*-retinol steady-state levels in mouse liver and testis (Belyaeva et al., 2018).

The conversion of retinaldehyde to RA is catalyzed by specific members of the aldehyde dehydrogenases (ALDHs) family. The cytosolic Class I ALDHs were initially termed ALDH1 in human, Ahd2 in the mouse, and RALDH or RalDHI in rat (Napoli, 2012). Mammalian genomes encode three different family members which are now termed ALDH1A1, ALDH1A2 and ALDH1A3, respectively. Most knowledge about embryonic RA synthesis comes from genetic dissection in single and compound knockout mice for these enzymes (Duester, 2008; Rhinn, Dollé, 2012). These studies clearly identified Aldh1A2 as a major enzymatic system for the production of RA as indicated by early embryonic death of null mice (Niederreither et al., 1999). Aldh1A3 is expressed in specific compartments later during mouse development (Mic et al., 2000). Aldh1A3-null mice display eye defects and die at birth due to respiratory distress consistent with the enzyme's role at later developmental stages (Vermot et al., 2003). In humans, Aldh1A3 gene mutations are associated with congenital microphthalmia (Yahyavi et al., 2013). Aldh1A1 knockout mice develop normally and are fertile (Fan et al., 2003b). These mice display several metabolic anomalies during adult life, e.g., they are resistant to diet-induced obesity (Ziouzenkova et al., 2007).

The conversion of retinol to RE is achieved by lecithin:retinol acyltransferase (LRAT) a vertebrate specific enzyme and has been molecularly cloned from several species. Studies in mice and zebrafish confirmed the enzyme's critical role in retinoid metabolism and vitamin

A homeostasis (Batten et al., 2004; Isken et al., 2007; Ruiz et al., 2001; Ruiz et al., 1999). Additionally, a diacylglycerol acyltransferase 1 (DGAT1) contributes to RE formation in some tissues (O'Byrne et al., 2005; Wongsiriroj et al., 2008). While DGAT1 uses coenzyme A as acyl donor for RE production. LRAT, a member of the ancestral NlpC/P60 thiol peptidase protein superfamily (Golczak et al., 2015; Pang et al., 2012), selectively transfers an acyl moiety from the *sn*-1 position of phosphatidylcholine (Golczak et al., 2015; MacDonald, Ong, 1988). The general structural motif of NlpC/P60 thiol peptidase is reminiscent of papain-like proteases and consists of a four-strand antiparallel β-sheet and three α-helices. The conserved catalytic residues Cys161, His60, and His72 define the active site. LRAT adopts an analogous catalytic strategy as thiol peptidases, whereby the deprotonated Cys161 serves as a nucleophile to attack the carbonyl carbon of an ester bond at the SN1 position of phosphatidylcholine, eventually leading to an acyl transfer and trans-esterification of retinol. LRAT-deficient mice lack liver and lung retinyl ester stores and are highly susceptible to vitamin A deficiency (Liu, Gudas, 2005; O'Byrne et al., 2005).

7.   Carotenoid and Retinoid transport to the eyes

The hydrophobic nature of carotenoids and retinoids limits their solubility and diffusion in the aqueous environment of the body. Therefore, carotenoids and retinoids are transported in the main lipoprotein classes of the body together with other lipids such as fatty acids, cholesterol, and fat-soluble vitamins. Additionally, animals have evolved specific binding proteins for the pigments. In vertebrates, three classes of retinoid-binding proteins named for their selectivity (i.e. retinol-binding proteins (RBP1–4), cellular RA-binding protein (CRABP1 and 2), and *cis*-retinoid-specific cellular retinal-binding proteins (CRALBP) carry retinoids within cells. Additionally, the interstitial retinol-binding protein 3 (IRBP) transports vitamin A between RPE and photoreceptors of the eyes (Liou et al., 1982; Liou et al., 1989). Vertebrate RBPs and CRABPs are members of lipocalin family of lipid-binding proteins. CRALBP, like insect Pinta, belongs to the Sec14 protein family of CRAL-Trio proteins (Widjaja-Adhi, Golczak, 2019). An isoform of glutathione-S-transferase, GSTP1, and a steroidogenic acute regulatory domain protein, StARD3, were described as zeaxanthin and lutein-binding proteins in the human retina (Bhosale, Bernstein, 2007; Li et al., 2011). The StARD3 protein belongs to the same protein class as the CBP from silkworm (*Bombyx mori*) (Sakudoh et al., 2007).

7. 1.   Vitamin A transport

For transport in lipoproteins, dietary vitamin A precursors are converted into RE and are incorporated into chylomicrons together with parent carotenoids and other dietary lipids (O'Byrne, Blaner, 2013; O'Byrne et al., 2005) (Figure 12). Chylomicron production occurs at the endoplasmic reticulum of enterocytes and requires surface proteins apolipoprotein B48 (apoB-48) and apolipoprotein A-IV. Microsomal triglyceride transport protein (MTP) catalyzes the lipidation of apoB-48 in the inner leaflet of the ER (Iqbal et al., 2008). The assembled pre-chylomicrons translocate to the Golgi apparatus where it acquires apolipoprotein A1. Mature chylomicrons are then secreted into the lymph and reach the circulation at the level of the subclavian vein. In the circulation, chylomicrons interact with lipoprotein lipase of peripheral tissues (He et al., 2018). The resulting chylomicron remnant

is taken up by the liver for processing of its remaining lipid cargo. Retinyl esters are cleaved into fatty acid and retinol by a yet not molecularly identified retinyl ester hydrolase(s) and transported to stellate (Ito) cells. Here, they are re-esterified by LRAT and stored in lipid droplets (D'Ambrosio et al., 2011; O'Byrne et al., 2005).

All-*trans*-retinol bound to RBP4 (holo-RBP4) is the major form of vitamin A in the fasting circulation. RBP4 is mainly produced in the liver (Thompson et al., 2017) and its secretion depends on the presence of vitamin A (Quadro et al., 1999) (Figure 12). In the blood stream, holo-RBP4 forms a ternary complex with transthyretin (Episkopou et al., 1993). The levels of this complex are homeostatic and not affected by fluctuations in dietary vitamin A supply. For many years, it remained elusive how retinol, in its transport form can cross cell membranes and enter the cytoplasm of target cells. In the mid 1970's, two groups independently reported evidence for the existence of a cellular receptor for RBP4. This molecule is present on the basolateral surface of bovine retinal pigment epithelium (RPE) cells (Heller, 1975; Heller, Bok, 1976) as well as on cells of the monkey's small intestine (Rask, Peterson, 1976). Thirty years after the biochemical description of the RBP4 receptor, the molecular nature of this protein was elucidated. This breakthrough was achieved using an elegant strategy that stabilized the fragile interaction between recombinant tagged RBP4 and its receptor by protein cross-linking followed by high affinity purification of the complex (Kawaguchi et al., 2007). The RBP4 receptor protein is encoded by the stimulated by retinoic acid gene 6 (*STRA6*) that has been previously identified as a RA-inducible gene (Bouillet et al., 1997). Cell culture studies revealed that STRA6 gene product fulfills all three criteria expected for a bona fide RBP4 membrane receptor: 1) RBP4 binds to this membrane protein 2) STRA6 mediates cellular uptake of vitamin A, and 3) STRA6 is expressed in many tissues in which the native receptor has been previously described including RPE, the choroid plexus of the brain, and the Sertoli cells of the testis (Kawaguchi et al., 2007). We then showed that knockdown of STRA6 in zebrafish embryos rendered the larval eyes vitamin A deficient, thus providing physiological evidence for STRA6's involvement in vitamin A transport (Isken et al., 2008).

Structural analysis revealed that STRA6 is assembled as an intricate dimer with 18 transmembrane helices (nine per protomer) and two long horizontal intramembrane helices interacting at the dimer core (Chen et al., 2016). The receptor complex displays a lipophilic cleft to which holo-RBP4 binds with high affinity (Chen et al., 2016; Kawaguchi et al., 2007). Studies in cell lines indicate that STRA6 facilitates the bidirectional flux of retinol between RBP4 and cells (Isken et al., 2008; Kawaguchi et al., 2011). Cellular accumulation of retinol is driven by esterification by LRAT (Amengual et al., 2012; Amengual et al., 2014b; Isken et al., 2008; Kawaguchi et al., 2011).

Mouse models for all major players of ocular vitamin A uptake from holo-RBP4 have been established. Early in life, *Stra6*$^{-/-}$ mice display very low ocular vitamin A levels and display highly reduced ERG responses (Amengual et al., 2014a; Ruiz et al., 2012). Blood and other tissues such as the lungs, fat, and liver display normal vitamin A levels when mice are bred and raised on vitamin A-rich diets (Amengual et al., 2014a; Berry et al., 2013). Ocular vitamin A levels increase with age when the mice are raised on diets rich in vitamin A (Amengual et al., 2014a; Kelly et al., 2016). A comparable phenotype has been reported for

RBP4-deficient mice (Amengual et al., 2014a; Quadro et al., 1999). These findings clearly indicate that RE in chylomicrons can compensate at least in part for the RBP4/STRA6 system when dietary supply with the vitamin is abundant. Genetic disruption of the *Lrat* gene renders mice blind because of the inability to acquire vitamin A from circulating RBP4 (Amengual et al., 2012) and to produce REs for visual chromophore production (Batten et al., 2004). LRAT-deficient mice also lack major retinoid stores of the body and are highly susceptible to dietary vitamin A deficiency (Liu, Gudas, 2005; O'Byrne et al., 2005).

The STRA6, RBP4, and LRAT dependent storage and distribution system for vitamin A provides the advantage to endure prolonged periods with little to no supplies of dietary vitamin A precursors. Particularly, the eyes benefit from this system. Dowling and Wald already described how vitamin A deprivation in rats successively affects retinoid levels in liver, blood, and eyes (Dowling, Wald, 1958). We observed in mice that the eyes display normal levels of vitamin A even after 20 weeks of vitamin A deprivation, though vitamin A stores in the liver and lung were nearly exhausted under this condition (Amengual et al., 2012). Mice only show significant losses of ocular vitamin A when they are subjected to eight months of dietary deprivation (Hu et al., 2011). In contrast, the eyes of STRA6-deficient mice are highly susceptible to dietary vitamin A deprivation, demonstrating the importance of STRA6 for ocular vitamin A homeostasis under this condition (Kelly et al., 2016). The preferred delivery of stored vitamin A to the eyes is explained by the regulation STRA6 gene expression which depends on tissue type and vitamin A status. *Stra6* is highly expressed in epithelia that constitute a blood tissue barrier, especially in the RPE, but also in Sertoli cells of the testis and ependymal cells of the choroid plexus (Amengual et al., 2014a; Berry et al., 2013; Kelly et al., 2016). In the eyes, which have the highest vitamin A requirement in the body, *Stra6* gene expression is independent from retinoid status (Amengual et al., 2012; Kelly et al., 2016). This explains why ocular retinoid homeostasis is maintained for prolonged times under dietary vitamin A deficiency (Amengual et al., 2012). In testis and brain, *Stra6* expression is higher than in most other tissues, but it is positively regulated by retinoids (Kelly et al., 2016). This regulation seemingly increases STRA6 expression in vitamin A sufficiency but reduces vitamin A consumption of these tissues when its dietary supply is limited, saving stored retinoids to be used in the eye. Other peripheral tissues such as lung and adipose tissues display very low expression of STRA6 and apparently depend on the delivery of postprandial dietary vitamin A in lipoproteins, which they can store. However, these tissues may play a role in quickly removing vitamin A from the circulation when it is present in excess through the sensitivity of *Stra6* expression to RA (Amengual et al., 2012; Amengual et al., 2014a). Accordingly, STRA6 expression is induced in peripheral tissues and blood holo-RBP4 levels decline when mice are treated with pharmacological doses of RA (Amengual et al., 2012). The coupling of STRA6-dependent cellular vitamin A uptake with LRAT activity provides an additional layer of regulation and fine tuning in tissues with a high vitamin A demand (Amengual et al., 2012). From these findings one deduces that STRA6 acts like a 'cellular faucet' which allows for a higher cellular uptake of circulating vitamin A than is possible when the uptake is solely reliant on passive diffusion. The elaborated regulation of STRA6 expression in peripheral tissues ensures proper distribution of vitamin A depending on tissue demand and availability of the nutrient.

In humans, 24 missense and nonsense mutations in the *STRA6* gene have been identified to cause a severe microphthalmic syndrome named Matthew-Wood syndrome (MWS) (Golzio et al., 2007; Pasutto et al., 2007). MWS is characterized by severe bilateral microphthalmia, often in combination with pulmonary dysplasia, cardiac defects, and diaphragmatic hernia, among other anomalies and malformations (Chassaing et al., 2009). The symptoms of MWS are consistent with the pivotal role of retinoids in mammalian embryonic development (Clagett-Dame, DeLuca, 2002), but are variable even within the same family ranging from isolated microphthalmia to fatal syndromes (Casey et al., 2011). Similarly, mutations in the *RBP4* gene can cause congenital eye malformations, including microphthalmia (Chou et al., 2015; Khan et al., 2016; Seeliger et al., 1999). Consistently, even within the same family large variations in the phenotypic manifestation of the *RBP4* mutation exist (Chou et al., 2015).

The severe ocular malformations in STRA6- and RBP4-deficient patients contrast the milder ocular phenotypes in mice. However, recent genetic analysis also revealed that mutations in the gene encoding the retinoic acid producing enzyme *ALDH1a3* cause microphthalmia in humans (Yahyavi et al., 2013), but a milder ocular phenotype in mice (Matt et al., 2005; Molotkov et al., 2006). Importantly, variability in the extra-ocular phenotype associated with *STRA6* mutations also may reflect maternal vitamin A status and delivery to the fetus. Studies in mice indicate that both dietary and stored vitamin A can be transported through the fetal-maternal-blood barrier (Quadro et al., 2005; Wassef, Quadro, 2011). Notably, dietary vitamin A restriction of *Rbp4* knockout mice results in malformations that resemble the birth defects of MWS patients (Quadro et al., 2005). Biochemical evidence has been provided that an RBP4 receptor is expressed in the placenta (Redondo et al., 2008). In mice it is unclear whether this receptor is STRA6 or the recently identified RBP-receptor 2 since both RBP4 receptors are expressed in this tissue (Alapatt et al., 2012; Kawaguchi et al., 2007; Kim et al., 2008).

7.2. Carotenoid transport

Circulating carotenoid levels are influenced by many factors, including individual genetics, health status, dietary intake, and vitamin A status (Bohn et al., 2017). Generally, their levels are correlated with serum triglyceride and cholesterol levels. Body composition is another major factor that affects the circulating levels of carotenoids. A number of studies have reported an inverse association between body mass index (BMI) and plasma carotenoid concentrations (Bohn et al., 2017; Wang et al., 2008). Further dissection of this association suggests that carotenoid plasma concentrations are inversely related not only to fat mass, but also to lean body mass. This indicates that non-fat tissues, such as muscle, may also serve as a reservoir for carotenoids (Bohn et al., 2017). Notably, people with anorexia nervosa have very high levels of plasma carotenoids, most likely due to high levels of mobilization in this catabolic condition (Curran-Celentano et al., 1985).

In the blood, carotenoids are not equally distributed among lipoprotein classes. Zeaxanthin and lutein exist in higher concentrations in HDL than in low density and very low density lipoproteins (Palczewski et al., 2016; Thomas, Harrison, 2016). Carotenoid content and composition is variable between tissues. The accumulation of macula pigments in the retina

indicate that specific transport mechanisms must exist for carotenoids. Studies in human retinal pigment epithelial cell lines indicate that scavenger receptor SR-B1 facilitates uptake of carotenoids from HDL (During et al., 2008; Thomas, Harrison, 2016). The retinal pigment epithelium cells acquire fat soluble vitamins and other nutrients to deliver them to the adjacent neuronal retina to support photoreceptor function. Carotenoid-binding proteins have been implicated in assisting macular pigment accumulation (Bernstein et al., 2016). However, given the very high levels of xanthophylls in the macula and the stoichiometry of their binding, it is unlikely that macular pigments predominantly exist in protein-bound form unless the carotenoid-binding proteins GSTP1 and StARD3 would be present in comparable amounts. β-Carotene in circulating LDL is absorbed by LDL receptor mediated endocytosis into the retinal pigment epithelium (Thomas, Harrison, 2016). Human RPE cells express relatively high levels of BCO1 (Yan et al., 2001), the vitamin A forming enzyme and local vitamin A synthesis may contribute to ocular retinoid homeostasis (Chichili et al., 2005).

8. Vitamin A metabolism in the eyes

Phototransduction, the process by which light energy is translated into a photoreceptor's electrical response, has long been at the forefront of sensory transduction and cell signaling studies (Hardie, Raghu, 2001; Lamb, Pugh, 2004). Vertebrate and invertebrate photoreceptors sequester their transduction machinery in specialized subcellular compartments. These compartments are characterized by the need to maximize the amount of light-absorbing membranes and harbor the phototransduction machinery. Insects photoreceptors display tightly packed microvilli, which are organized in a cylindrical rhabdomere. Vertebrate rods achieve this with stacks of membranous discs that are internalized in the rod outer segment. The outer segment is separated from the rest of the cell by a short ciliary stalk. Vertebrate cones display invaginations of the plasma membrane (Hofmann, Palczewski, 2015).

The elucidation of the biochemical steps involved in phototransduction led to the discovery and characterization of visual G protein signaling and culminated in the determination of the structure of the heptahelical transmembrane receptor rhodopsin (Palczewski, 2006; Palczewski et al., 2000). These transmembrane proteins activate heterotrimeric G proteins and are involved in a broad range of physiological processes throughout the body, where they respond to a wide variety of chemical messengers including hormones, neurotransmitters, odorants, and food ingredients. Visual pigments comprise one class of G protein-coupled receptors and consist of an integral transmembrane protein (opsin) and a covalently bound retinylidene chromophore (von Lintig et al., 2010). Other chromophore-binding G protein coupled receptors, such as melanopsin, regulate circadian rhythms, suppress pineal melatonin, modify locomotor activity, and modulate pupil size in the mammalian eyes (Foster, Hankins, 2002).

The 11-*cis*-diastereomer of the chromophore binds by a Schiff-base linkage to a membrane-embedded Lys residue in the opsin molecule to form functional visual pigments. In vertebrates, absorption of light triggers a geometric isomerization of the chromophore that converts rhodopsin into an activated state termed Meta II. Meta II is catalytically active and binds transducin (Gt), a photoreceptor-specific G protein, thereby initiating a signal-

amplifying cascade involving cGMP that results in plasma membrane hyperpolarization. In Drosophila, rhodopsin activation and binding of Gt initiates phosphoinositide signaling, culminating in the opening of transient receptor potential (TRP) channels and depolarization of the photoreceptor cell membrane.

To regenerate chromophore after photo-bleach, animals have evolved two strategies: In bistable visual pigments of invertebrates, the chromophore remains covalently bound to the opsin and absorption of a second photon isomerizes the all-*trans*- back to the 11-*cis*-configuration of chromophore (von Lintig, 2012; Wang, Montell, 2007). In ciliary photoreceptors of vertebrates, the visual pigments decay following photo-activation into opsin and free all-*trans*-retinal. To restore light sensitivity, the photoproduct must be regenerated by a multi-step enzymatic pathway, named the visual cycle (Kiser et al., 2014; Wald, 1968).

Research in our and other laboratories has challenged the strict classification into light-dependent and independent mechanisms for chromophore regeneration in the two main animal groups. In *Drosophila*, an enzyme-based pathway was discovered for the production and regeneration of chromophore (Oberhauser et al., 2008; Voolstra et al., 2010; Wang et al., 2010; Wang et al., 2012). In mice, it was observed that chromophore production is accelerated by light (Wenzel et al., 2005b). In keeping with this finding, a blue light-dependent photoreversal of rhodopsin has been described in the literature (Grimm et al., 2000; Grimm et al., 2001; Hubbard, Kropf, 1958; Williams, 1964). Additionally, evidence for blue light-dependent isomerization of phosphatidylamine-chromophore conjugates (Kaylor et al., 2017) and RPE-retinal G-protein–coupled receptor (RGR)-opsin-dependent mechanisms have been reported in the mammalian eyes (Chen et al., 2001; Diaz et al., 2017; Morshedian et al., 2019; Zhang et al., 2019).

8. 1. Chromophore production and recycling in insects

Retinoids are mainly required for vision in *Drosophila* (Harris et al., 1977). This characteristic is demonstrated through the significant reduction of the retinoid content in *sine oculis* fly mutants that lack compound eyes (Voolstra et al., 2010) (Figure 13 A,C). Transcriptomic analysis of this mutant also revealed an eye-enriched expression of major carotenoid and retinoid processing enzymes, including NinaB, Pinta, and the retinol dehydrogenases Pdh and RdhB (Xu et al., 2004) (Fig. 13 B). The latter three proteins are enriched in pigment cells, suggesting that this cell type displays an analogous function in chromophore metabolism to the RPE in vertebrate eyes (Wang, Montell, 2005; Wang et al., 2010; Wang et al., 2012).

In fly photoreceptors, the opsin is synthesized at the endoplasmic reticulum, combined with chromophore, transported through the Golgi apparatus, and inserted into rhabdomeric membranes (Schopf, Huber, 2017). This synthesis requires the 11-*cis*-diastereomer of chromophore because the opsin is degraded in its absence (Huber et al., 1994; Oberhauser et al., 2008; Ozaki et al., 1993). Isono and colleagues observed that carotenoids but not retinoids promoted visual pigment synthesis in flies that were reared in darkness (Isono et al., 1988). The biochemical basis for this observation was provided by the identification of the *ninaB* gene and the characterization of the encoded enzyme. Missense and nonsense

mutations in *ninaB* gene disrupt the *de novo* synthesis of chromophore and visual pigment (von Lintig et al., 2001). Supplementation of 11-*cis*-retinal but not all-*trans*-retinal restores visual pigment synthesis in the dark-reared *ninaB* mutant. Additionally, supplementation of carotenoids and/or 11-*cis*-retinal but not all-*trans*-retinal promoted visual pigment synthesis in wild type flies which were reared on vitamin A-deprived chow in the dark (Oberhauser et al., 2008). Analysis of the NinaB enzyme showed that it catalyzes an isomer-oxygenase reaction which converts a carotenoid into an 11-*cis*-retinal and all-*trans*-retinal product in 1:1 molar ratio (Figures 5 and 13 D) (Chai et al., 2019; Oberhauser et al., 2008). The unique reaction sequence follows a dioxygenase mechanism with a carbocation/radical intermediate (Babino et al., 2016). Pinta protein is required for transport of the 11-*cis* diastereomer of the chromophore to photoreceptor cells. The *pinta* mutant displays highly reduced visual pigment content in photoreceptors (Wang, Montell, 2005), though the mutant flies can produce the 11-cis-diastereomer of the chromophore (Voolstra et al., 2010).

The all-*trans*-retinal cleavage product of the NinaB-catalyzed conversion of a carotenoids is further metabolized by a light-dependent pathway. This pathway involves a (blue) light-dependent isomerization reaction that awaits molecular description (Ozaki et al., 1993; Schwemer et al., 1984; Smith, Goldsmith, 1991). The existence of this pathway explains why light-reared flies can synthesize visual pigments from supplemented all-*trans*-retinal as precursor.

In addition to NinaB and Pinta, a retinol dehydrogenase, encoded by the *rdhB* gene is required for visual pigment production in flies (Wang et al., 2012). An oxidoreductase, encoded by the *ninaG* gene, has been also implicated in this pathway. The *ninaG* mutant shows highly reduced content of Rh1, the major rhodopsin of the fly eyes (Ahmad et al., 2006; Sarfare et al., 2005). *NinaG* mutant flies accumulate 3-hydroxy-retinol and the NinaG protein has been implicated in the conversion of 3(R)-hydroxy-retinol into the 3(S)-enantiomer (Sarfare et al., 2005). A pathway for the conversion of all-*trans*-retinal into all-*trans*-3(S)-hydroxy-retinal has been described in flies, and evidence exists that Drosophila utilizes this enantiomer as chromophore (Seki et al., 1998).

Insect visual pigments are bistable. Once bound to the opsin, the chromophore remains covalently bound after a bleach. Absorption of a second photon isomerizes the all-*trans*-photoproduct back to 11-*cis*-diastereomer and restores light sensitivity to the visual pigment (von Lintig, 2012; Wang, Montell, 2007). Therefore, it was generally not assumed that flies exhibit a regeneration pathway for chromophore as commonly found in vertebrates. However, biochemical evidence from blow flies indicate the existence of a renewal pathway for rhodopsin (Schwemer, 1984). In Drosophila, it has been observed that photo-bleached visual pigments removed from rhabdomeric membranes under constant illumination conditions (Satoh, Ready, 2005; Wang et al., 2010). The analysis of the Drosophila *pdh* mutant provided genetic evidence that the chromophre of internalized visual pigments is regenerated through an enzymatic pathway (Wang et al., 2010) (Figure 13 D). The *pdh* gene encodes an enzyme which belongs to the short-chain dehydrogenase/reductase (SDR) family. The *pdh* mutant is characterized by a progressive light-dependent loss of visual pigments that is accompanied by degeneration of photoreceptors. The light-dependent degeneration of photoreceptors in Pdh-deficiency is likely explained by the toxicity of opsin-

unbound chromophore. This assumption is based on experiments that showed that retinal degeneration of opsin-deficient fly mutants is prevented in chromophore-deficient photoreceptors. Additionally, it has been shown that free retinaldehyde but not retinol can induce oxidative stress and apoptosis in *Drosophila* S2 cells (Voolstra et al., 2010). Accordingly, dark-reared *pdh* mutant flies are protected against visual pigment loss and photoreceptor degeneration. Similarly, photoreceptor degeneration can be prevented in *pdh* mutants by the expression of human *RDH12*. This retinal dehydrogenase reduces the aldehyde form of the photoproduct into the corresponding alcohol (Haeseleer et al., 2002; Wang et al., 2010). Biochemical analysis confirmed that recombinant Pdh, similar to RDH12, catalyzes an interconversion of all-*trans*-3-hydroxy-retinal to all-*trans*-3-hydroxy-retinol (Hofmann et al., 2016). The all-*trans*-3-hydroxy-retinol can then be recycled to 11-*cis*-3-hydroxy-retinal by a pathway that depends on RdhB and light (Wang et al., 2012). The ability to recycle chromophore trough this pathway enables flies to maintain normal chromophore levels under conditions of prolonged dietary vitamin A deprivation (Wang et al., 2010).

8. 2. The visual cycle of vertebrates

In the disc membranes of rod outer segments (ROS) of the vertebrate eye, rhodopsin exists as an integral membrane protein and the chromophore is covalently bound via a Schiff base. Light induces a geometric isomerization of the protein-bound chromophore to initiate visual phototransduction (Arshavsky et al., 2002). Hydrolysis of the Schiff base linkage by bulk water entering from the cytoplasmic side liberates the all-*trans*-retinal photoproduct (Jastrzebska et al., 2011). The photoproduct must be converted back to chromophore to sustain vision. Individual steps in this cyclic biochemical pathway have been described by Wald and are now understood in biochemical and molecular detail (for an overview see Figure 14). Mutations in genes encoding these proteins are associated with inherited blinding diseases, including Leber congenital amaurosis and various forms of retinitis pigmentosa (Kiser, Palczewski, 2016; Thompson, Gal, 2003). Furthermore, the generation of knockout mouse models in these genes have confirmed the biochemical role of the encoded proteins and provided models to establish therapeutic intervention strategies (Garafalo et al., 2019; Sears et al., 2017).

After its release from rhodopsin, all-*trans*-retinal enters the disk lumen and must be transferred to the cytosol by the ATP-binding cassette transporter 4 (ABCA4) (Molday et al., 2000; Molday et al., 2009; Tsybovsky et al., 2010). For this transport, all-*trans*-retinal forms conjugates with phosphatidylethanolamine, an abundant membrane lipid in disk membranes. ABCA4 catalyzes N-retinylidene-phosphatidylethanolamine import into the cytosol in an ATP-dependent fashion (Quazi et al., 2012; Tsybovsky et al., 2013). This transporter also plays an important role in the control of chromophore homeostasis in the dark-adapted photoreceptors (Quazi, Molday, 2014).

In the cytoplasm, the photoproduct is converted to all-*trans*-retinol in a reaction catalyzed by RDHs (Haeseleer et al., 1998; Rattner et al., 2000). Two enzymes, Rdh8 in photoreceptor outer segments and RDH12 in photoreceptor inner segments employ NADPH as a cofactor and are mainly responsible for catalyzing this reaction in mouse rod photoreceptors (Maeda

et al., 2005). However, the redundancy of retinal reductase activity shown in mice suggests that photoreceptors may contain additional RDHs aside from RDH12 and RDH8 (Maeda et al., 2007a). This redundancy could be due to the need for a large enzymatic capacity to convert the chemically reactive aldehyde group of the photoproduct to the corresponding alcohol.

Impairment of all-*trans*-retinal clearance can cause severe retinal pathologies. The aldehyde group of the photoproduct can form adducts with primary amino groups such as phosphatidylethanolamine to form bisretinoids such as A2E. Ocular accumulation of A2E together with A2E-mediated redox reactions have been implicated in the pathology of eye diseases such as Stargardt disease and the age related macular degeneration (Sparrow, Boulton, 2005; Sparrow et al., 2020; Zhou et al., 2006). The importance for rapid clearance of the photoproduct is demonstrated by the pathological consequences of mutations in RDH12 and ABCA4 in humans (Allikmets et al., 1997; Janecke et al., 2004). Notably, *ABCA4* knockout mice accumulate bisretinoids such as A2E (Mata et al., 2001; Radu et al., 2004). Retinal pathology is augmented when *ABCA4* and *RDH8* genes are genetically disrupted together in mice (Maeda et al., 2008). In addition of A2E formation, acute all-*trans*-retinal toxicity may contribute to the retinal pathology in the compound mutant (Maeda et al., 2009) by the formation of retinal-adducts with other cellular molecules, including proteins and ribonucleotides (Chen et al., 2012; Maeda et al., 2012).

All-*trans*-retinol formed in rod outer segments is transported to the RPE where it is esterified. This process is facilitated by IRBP that binds retinoids in the extracellular space. Missense mutations in the human *IRBP* gene have been identified and can cause autosomal recessive retinitis pigmentosa (den Hollander et al., 2009). CRBP1 is needed for the transport of all-*trans*-retinol within RPE cells (Edwards, Adler, 1994; Redmond et al., 1985). CRBP1 has recently identified as putative novel target for visual cycle modulation. Abnormal cannabidiol is a nonretinoid inhibitor of CRBP1, reduces the flux through the visual cycle, and can ameliorate consequences of bright light damage to the retina (Silvaroli et al., 2019).

The major ester synthase in RPE is LRAT (Batten et al., 2004; Saari, Bredberg, 1988). This membrane-anchored enzyme plays a central role in retinoid metabolism of the RPE. It is required for the STRA6-dependent uptake of all-*trans*-retinol from circulating holo-RBP4 (Amengual et al., 2012), helps to form oil droplet-like structures, named retinosomes (Imanishi et al., 2004), and produces RE as substrate for the retinoid isomerase RPE65. This latter enzyme catalyzes the transformation of all-*trans*-retinyl esters to 11-*cis*-retinol and palmitate (Jin et al., 2005; Moiseyev et al., 2005; Redmond et al., 2005a). The product of this isomerization reaction is subsequently oxidized in the final catalytic step of the visual cycle to 11-*cis*-retinal. Enzymatic activities of RDH5, RDH10 and RDH11 are mainly responsible for this reaction (Haeseleer et al., 2002; Sahu et al., 2015) but additional 11-*cis*-RDHs may participate within the RPE (Maeda et al., 2007b). Newly synthesized 11-*cis*-retinal is protected by binding to CRALBP which mediates its transport back to photoreceptor ROS where the chromophore covalently binds to opsin, thereby completing the visual cycle (Saari, Bredberg, 1987). Disrupting the enzymatic steps of chromophore regeneration in the RPE, especially those involving LRAT and RPE65, has severe

pathological consequences on retinal health (Marlhens et al., 1997; Thompson, Gal, 2003). The resulting chromophore deficiency causes slow progressive death of rods that is attributed to continuous activation of visual phototransduction by unliganded opsin (Woodruff et al., 2003). Moreover, disordered processing and transport of cone visual pigments lacking bound-chromophore leads to very rapid cone degeneration in knockout mouse models (Zhang et al., 2008).

8.2.1   Modifications of the canonical visual cycle in diurnal animals—Mice are nocturnal animals and mainly rely on scotopic vision that is mediated by rods. Daylight vision is mediated by cones and is critical for visual acuity and color discrimination. Cone response kinetics differ significantly from rods. Following light flashes that generate similar membrane currents, cones recover sensitivity approximately 10-fold faster than rods (Perry, McNaughton, 1991). Moreover, the rod photo-response is saturated at photoisomerization rates above 500 per second (Baylor et al., 1984) but cones remain responsive to light at photoisomerization rates up to 1,000,000 per second (Schnapf et al., 1990).

In diurnal mammals, the different characteristics of cones and rods may necessitate specific mechanism(s) for chromophore regeneration to avoid competition for the unique chromophore, 11-*cis*-retinal. The competition between these two types of photoreceptors has been convincingly demonstrated in the retina of the R91W *Rpe65* mutant mouse that can produce only minute amounts of chromophore. Due to the higher number of rods and the instability of cone opsin, cones suffer from chromophore deficiency and degenerate in the retina of this mouse mutant (Samardzija et al., 2009).

Evidence exists that modifications of the canonical visual cycle may help diurnal animals to cope with the increased demand for visual pigment regeneration under day light conditions. Moiseyev and colleagues showed that chicken RPE65 display significant increased isomerase efficiency when compared to its mouse counterpart (Moiseyev et al., 2008), thus allowing faster chromophore regeneration. Another hallmark of eyes with high resolution color vision, including the human eyes, is the existence of 11-*cis*-RE (Mustafi et al., 2016). How this vitamin A metabolite is synthesized and whether it can support cone vision was recently investigated in the zebrafish (*Danio rerio*) eyes (Babino et al., 2015b). The eyes of the zebrafish larva are amenable for genetic and pharmacological interventions and have been successfully used to study photoreceptor development and function (Fleisch, Neuhauss, 2010). These studies showed that the larval eyes express key components of the canonical visual cycle, including Stra6, Lrat, Rpe65, and Cralbp (Fleisch et al., 2008; Isken et al., 2008; Isken et al., 2007; Schonthaler et al., 2007). Similar to diurnal mammals, 11-*cis*-REs exist in the RPE of dark-adapted eyes of larval and adult zebrafish (Figure 15A, B). This was demonstrated by biochemical analyses as well as by two-photon microscopy of the larval eyes (Palczewska et al., 2010) (Figure 15C). The levels of 11-*cis*-REs are comparable to that of all-*trans*-REs, also known to be present in the RPE as storage pools for chromophore production. Wild type and cone-only mutant larvae were employed to test whether 11-*cis*-REs contribute to cone vision with a recovered sensitivity approximately 10-fold faster than rods (Baylor et al., 1979; Perry, McNaughton, 1991). The experiments revealed that after an initial drop in 11-*cis*-retinal, levels of the chromophore achieved a steady-state, while levels of 11-*cis*-REs continuously diminished to nearly undetectable levels after prolonged

bleaching time. This finding indicated that steady state levels of the 11-*cis*-retinal were achieved at least in part though hydrolysis of 11-*cis*-REs. Interestingly, previous studies have reported that hydrolysis of 11-*cis*-RE also occurs in homogenates of human retinal epithelial cells (Blaner et al., 1987).

To elucidate the pathway by which 11-*cis*-REs is generated in the zebrafish RPE, retinyl amine, a potent inhibitor of the RPE65 isomerase of the visual cycle, was used (Schonthaler et al., 2007). This approach showed that the inhibitor prohibited the regeneration of 11-*cis*-REs under dark conditions in both wild-type and cone-only zebrafish larvae (Babino et al., 2015b).

These experiments indicate that 11-*cis*-REs are synthesized in the dark and provide a pool of 11-*cis*-retinol that can be utilized under bright light luminance (for an overview see Figure 16), when the relatively slow RPE65 catalyzed isomerization reaction becomes rate limiting (Lyubarsky et al., 2005; Wenzel et al., 2005b). Under this condition, 11-*cis*-REs are hydrolyzed to produce 11-*cis*-retinol and shuffled to cones, likely by binding to Cralbpa (in the RPE) and Cralbpb (in Müller glia) (Fleisch et al., 2008; Schonthaler et al., 2008). Here it would subsequently be oxidized by *cis*-stereoisomer specific retinal dehydrogenases into 11-*cis*-retinal. This 'dark-generated' pool of chromophore precursor elegantly and efficiently supplies cones of zebrafish larvae with chromophore under bright light illuminance. It remains to be clarified whether this pathway also exists in diurnal mammals.

8.3. Light-dependent regeneration pathways for chromophore in the vertebrate retina

Bleached rhodopsin may be regenerated, not only in the dark through enzymes, but also photochemically. *In vitro*, visible light of short wavelength (blue light) can regenerate rhodopsin by a process called photoreversal of bleaching and it has been demonstrated that it occurs in the living rat eye (Grimm et al., 2000; Grimm et al., 2001; Hubbard, Kropf, 1958; Williams, 1964). Recently, it was shown that blue light illumination of a bleached mouse retina results in a significant increase in 9-*cis*- and 11-*cis*-retinal, thus providing further evidence for light-dependent re-isomerization of chromophore in photoreceptors. Additionally, it was shown that the photoproduct all-*trans*-retinal can form conjugates with PE in photoreceptors that, when illuminated with blue light (450 nm), are converted to the corresponding 11-*cis*-N-ret-PE. The quantum efficiency of this enzyme-independent photoconversion is similar to rhodopsin and blue light (450 nm) induced visual pigment regeneration occurs in the living mouse retina (Kaylor et al., 2017).

Additionally, light dependent isomerases may exist that interconvert retinal diastereomers. In mollusks an opsin protein known as retinochrome and a retinoid-binding protein, which are phylogenetically related to RPE-retinal G-protein-coupled receptor (RGR)-opsin and to CRALBP are involved in this pathway (Albalat, 2012). RGR has been implicated as constituent of a photic visual cycle in vertebrates as well (Yang, Fong, 2002). Evidence has been provided that RGR contributes to chromophore regeneration in *Rpe65*^{−/−} but not in *Rpe65*^{−/−}*Rgr*^{−/−} compound knockout mice (Van Hooser et al., 2002). However, the relatively low rate of 11-*cis*-retinal synthesis casted doubts on the physiological relevance of this finding (Hao, Fong, 1999). Recent biochemical studies revived the role of RGR in visual chromophore regeneration (Zhang et al., 2019). Using bovine RPE protein extracts, the

Palczewski laboratory demonstrated high-level production of 11-*cis*-retinal in RPE membranes upon narrow band green light illumination (for an overview see Figure 14). The activity increased with protein concentration and displayed substrate saturation. The presence of CRALBP stimulated 11-*cis*-retinal production in these assays. Using specific inhibitors for RPE65, the researchers demonstrated that the activity was associated with RGR. Most importantly, recombinant RGR exhibited the same activity in cell-based and cell-free enzyme assays. Site directed mutagenesis and biochemical testing revealed that the isomerase reaction involves a Schiff' base linkage of the all-*trans*-retinal substrate at Lys255 of RGR. Single-cell RNA-Seq analysis of the retina and RPE tissue showed that RGR is expressed in human and bovine RPE and Müller glia cells, whereas mouse RGR is expressed in RPE but at much lower levels in Müller glial cells (Zhang et al., 2019). The latter finding contrasts studies in mice which indicate that RGR in concert with a retinol dehydrogenase enzyme (Xue et al., 2017), contributes to cone visual pigment regeneration in Müller glia cells (Morshedian et al., 2019).

However, RGR also was associated with retinyl ester metabolism in the RPE and implicated as activator of RPE65 activity (Radu et al., 2008; Wenzel et al., 2005b). In the latter study, rhodopsin regeneration was found to be attenuated in $Rgr^{-/-}$ mice in both light and darkness. Additionally, it was shown that RGR regeneration was accelerated by light in an RGR-independent fashion (Wenzel et al., 2005b). Further research is needed to clarify the role of RGR in visual pigment regeneration in vertebrates.

8.4. An alternative retinoid cycle in the inner retina?

Studies in lower vertebrates showed that cone, but not rod, visual pigment regenerates independent of the RPE (reviewed in, (Fleisch, Neuhauss, 2010)), indicating that additional pathways for chromophore regeneration exist. Electrophysiological studies provided evidence that an intra-retinal pathway for cone visual pigment regeneration also exists in mice and humans (Wang, Kefalov, 2009). In this pathway, IRBP specifically delivers 11-*cis*-retinal to cone photoreceptors (Parker et al., 2011). Biochemical studies provided evidence that pharmacological inhibition of RPE65 impair but not completely abolish cone vision (Kiser et al., 2018). Furthermore, Müller glia is seemingly involved in this pathway because the genetic disruption of CRALBP expression in these cells impairs cone vision in the zebrafish (Fleisch et al., 2008) and mouse retina (Xue et al., 2015).

In the early 2000s, the Travis laboratory analyzed enzyme activities in the neuronal retina of chicken and ground squirrel and proposed an alternative visual cycle (Mata et al., 2002). In this alternative visual cycle, the photoproduct all-*trans*-retinal is reduced to all-*trans*-retinol by RDH8 in cone photoreceptors and IRBP-dependently transferred to Müller glial cells (Parker et al., 2011; Tang et al., 2013). All-*trans* retinol is isomerized to 11-*cis*-retinol by an isomerase in Müller glial cells (Mata et al., 2005). The endothermic isomerase reaction is driven by an AcylCoA-dependent esterification. An 11-*cis*-RE hydrolase in conjunction with a photoreceptor specific 11-cis-RDH synthesizes 11-*cis*-retinal and completes the cycle (Mata et al., 2002). Evidence for this pathway comes from zebrafish larvae in which gene knockdown and pharmacological inhibition of RPE65 does not significantly affect cone vision (Schonthaler et al., 2007; Ward et al., 2020). The putative alternative retinol

isomerase later was identified as dihydroceramide desaturase-1 (DES1) (Kaylor et al., 2013). Recombinant DES1 displays retinoid isomerase activity in the presence of appropriate cofactors though the enzyme was originally characterized in sphingolipid metabolism. DES1 was shown to be expressed in Müller glial cells and to be associated with CRALBP (Kaylor et al., 2013). Because DES1 catalyzes an equilibrium reaction between various retinol diastereomers (Kaylor et al., 2013), a multifunctional O-acyltransferase (MFAT) is proposed to drive the isomerization reaction by sequestering 11-*cis*-retinol in the form of its fatty acid ester (Kaylor et al., 2014). MFAT is allosterically regulated by 11-*cis*-retinoids (Arne et al., 2017). In this positive-feedback regulation, esterification rates for 9-*cis*, 13-*cis*, or all-*trans* retinols decrease and enable preferential synthesis of 11-*cis*-RE (Arne et al., 2017).

Efforts to provide genetic evidence for the alternative isomerase in the cone visual cycle were largely unsuccessful. A Des1-deficient zebrafish larva displays normal cone responses (Ward et al., 2020). Moreover, a Müller cell specific *Des1* knockout in mice revealed no changes in cone photoreceptor responses (Kiser et al., 2019). In line with these studies, gene knockout of RPE65, the retinoid isomerase of the canonical visual cycle, abolishes cone and rod photoreceptor response in mice (Redmond et al., 1998). Some residual light sensitivity of young *RPE65* knockout mice could be attributed to rod photoreceptors (Seeliger et al., 2001) and are mediated by 9-*cis*-retinal and isorhodopsin (Fan et al., 2003a). Additionally, studies of *Nrl*$^{-/-}$ mice with a cone only retina demonstrated that Rpe65 is critical for cone vision in mice (Wenzel et al., 2007). Thus, the studies for an alternative light-independent visual cycle in the retina remain inconclusive.

8.5. Putative interaction between carotenoids and the visual cycle

Many vertebrates accumulate significant amounts of carotenoids in their retina. In birds, carotenoids and long-chain apocarotenoids act as filters, substantially modifying light detection by cone photoreceptors, thereby fine-tuning spectral sensitivity and improving color discrimination. Macular pigments in the primate eyes reduce the adverse impact of light scattering and chromatic aberration, thereby optimizing contrast sensitivity of the retina (Hammond et al., 2013). These macular pigments also absorb short-wavelength light and scavenge reactive oxygen species (Bernstein et al., 2016; Hammond et al., 2013), both hallmarks of light damage of the retina (Hunter et al., 2012; Maeda et al., 2012; Wenzel et al., 2005a). We recently observed in wild type mice that ultraviolet light (405 nm) illumination of bleached photoreceptors can trigger a geometric isomerization of the photoproduct that results in the accumulation of 9-*cis*- and 13-*cis*-retinal (Widjaja-Adhi et al., 2018). These aberrant *cis*-retinal diastereomers persist for prolonged time in the retina and their formation is associated with morphological damage of the tissue. Notably, A2E concentration is inversely related to the concentration of carotenoids in patients (Bhosale et al., 2009) and studies in quails indicate that carotenoids can reduce A2E levels in the eyes (Bhosale et al., 2009). Additionally, macular pigments prevent photo-oxidation of A2E in cell culture models (Kim et al., 2006). However, research about the pigments beneficial roles in vision is limited because mice, the most common and available ophthalmologic animal model, do not accumulate these compounds (Bernstein et al., 2016). To overcome this hurdle, we employed *Bco2* knockout mice (Amengual et al., 2011b), which accumulate significant amounts of xanthophylls in their eyes (Amengual et al., 2011b; Li et al., 2014;

Widjaja-Adhi et al., 2015). We observed that already relatively low amounts of carotenoids reduced the levels of aberrant *cis*-retinal diastereomers after blue light bleaching and alleviated the associated retinal pathology (Widjaja-Adhi et al., 2018). An *in vitro* test system revealed that zeaxanthin can efficiently prevent blue light-induced geometric isomerization of all-*trans*-retinal in the test tube.

The observed interaction between carotenoids and retinoids may also explain why nocturnal animals such as mice naturally don't accumulate carotenoids in their retina. Their light absorbing properties would reduce sensitivity of visual pigments under dim light conditions by absorbing light in the range of 400 to 500 nm. Notably, owls have been reported to have pale oil droplets in the retina that lack carotenoids whereas diurnal birds accumulate these compounds (Bowmaker, Martin, 1978). The interaction between carotenoids and retinoids also may be considered in the recently proposed pathway for blue light-dependent chromophore regeneration (Kaylor et al., 2017; Morshedian et al., 2019) because the light absorbing properties of carotenoids would likely interfere with these processes. This interaction would be even more pronounced in the primate retina where macula carotenoids exist in very high concentrations.

Another notable interaction between carotenoids and the visual cycle is the putative involvement of RPE65 in macular pigment metabolism (Shyam et al., 2017a). Previously, it was shown that meso-zeaxanthin and RPE65 expression concomitantly appear in the RPE of the developing chicken embryos (Gorusupudi et al., 2016). Supply of lutein to human embryonic kidney (HEK293T) cells expressing recombinant RPE65 revealed turnover of lutein into meso-zeaxanthin. Furthermore, primary cultures of chicken RPE, also accumulated meso-zeaxanthin when incubated with lutein in a dose- and time-dependent manner. To test whether the reaction occurs in live animals, a small molecule inhibitor of RPE65 was injected into the yolk of chicken embryos and demonstrated to block *meso*-zeaxanthin synthesis. Thus, this studies show that RPE65 is involved in the synthesis of this unique ocular carotenoid. Further research will be required to understand the molecular basis of the unusual catalytic promiscuity of this visual cycle enzyme and its impact on retinoid metabolism of the eyes.

9. Conclusions and future perspectives

Much progress has been made in elucidating the metabolism of carotenoids related to vision. Comparative analyses in different animal classes has identified evolutionarily, well conserved key components in this metabolism. Mutations in their corresponding genes impair carotenoid metabolism and induce various visual pathologies in animal models. In humans, these genes are afflicted by mutations that can cause a wide spectrum of blinding diseases. The better understanding of the pathology of these diseases has paved the way for the development of therapeutic interventions. These include gene therapies and pharmacological interventions. Small molecules targeting visual cycle proteins have been developed and are in clinical tests. Just recently, small molecules targeting retinoid-binding protein CRBP1 have shown promising results in mice. An ancestral family of CCDs catalyzes the oxidative cleavage and geometric isomerization of carotenoid double bonds. The specific enzymatic properties of these enzymes are critical for chromophore production

and its quality control. Studies in mouse models revealed that carotenoid metabolism is regulated on demand for vitamin A supply. This mechanism helps to prevent retinoid deficiency when the dietary supply is scant and prevents excess during times of food surplus. The emerging knowledge about host and environmental factors that affect carotenoid metabolism asks for careful reevaluation of existing intake recommendations for these nutrients and further studies of their eye protecting roles. The generation of mice that accumulate significant amounts of carotenoids in the retina will allow testing of the pigment's ability to protect the retina and retinal pigment epithelium against damaging illumination and oxidative stress. These analyses should clarify putative interaction between carotenoids and the visual cycle since evidence exists that carotenoids can filter blue light that triggers geometric isomerization of the photoproduct and prevent the chemical modifications of visual cycle intermediates. A specific and selective transport system for stored vitamin A has been identified that helps the eyes maintain retinoid homeostasis even during times of prolonged dietary vitamin A deprivation. In STRA6-deficient mice the ocular retinoid content can be manipulated by dietary intervention thus allowing to study the consequences of conditions of mild to severe vitamin A deficiency that is widespread in children many parts of the world. With regards to the visual cycle, the question whether cones and rods rely on different regeneration pathways for chromophore is still a matter of debate. The role of photic regeneration pathways need to be studied in further details. Alternatively, modifications of the canonical visual cycle may well serve the higher demand for chromophore of cones. For this endeavor, researchers may strike out into new directions and take advantage of vertebrate models with cone-rich retinas. The zebrafish and chicken are promising models for such research and the newly established CRISPR-Cas technology offers opportunities to scrutinize their chromophore metabolism. Fostering our knowledge about the chemistry and biology of carotenoids in vision is and continues to be of substantial research interest. A better understanding of the involved molecular factors will aid in the development of nutritional intervention strategies to improve health in neonatal and adult life.

Acknowledgments

Abbreviations

AMD	age related macular degeneration
ADH	alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
BCO1	gene encoding β-carotene-15,15′-dioxygenase

BCO2	gene encoding β-carotene-9,10'-dioxygenase
CCD	Carotenoid Cleavage Dioxygenases
CD36	Cluster of Differentiation 36
CRALBP	Cellular retinal binding protein
Cyp26a1	Cytochrome P450 Family 26 A1
DES1	sphingolipid δ4 desaturase
IRBP	interstitial retinol-binding protein
ISX	Intestine-Specific Homeobox Transcription factor
LCA	Leber congenital amaurosis
LRAT	lecithin: retinol acyl transferase
MFAT	multifunctional *O*-acyltransferase
NinaD	neither inactivation nor afterpotential mutant D
NinaB	neither inactivation nor afterpotential mutant B
PDH	Pigment Cell enriched Dehydrogenase
RPE65	Retina Pigment Epithelium-Specific Protein 65kDa
Pinta	prolonged depolarization afterpotential is not apparent
SR-B1	Scavenger Receptor Class B, Member 1
STRA6	Stimulated by Retinoic Acid 6
RALDH1–3	Retinal Dehydrogenase 1–3
RAR	retinoic acid receptor
RBP (1–4)	retinol-binding protein
RDH	retinol dehydrogenase
RGR	RPE-retinal G-protein-coupled receptor (RGR)-opsin
RXR	retinoid X receptor
SDR	short chain dehydrogenase/reductase

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Highlights

Carotenoid and apo-carotenoid functions.

Carotenoid absorption and its homeostatic regulation.

Carotenoid bioconversion to vitamin A.

Transport and body distribution of carotenoids and retinoids.

Chromophore metabolism in different animal classes.
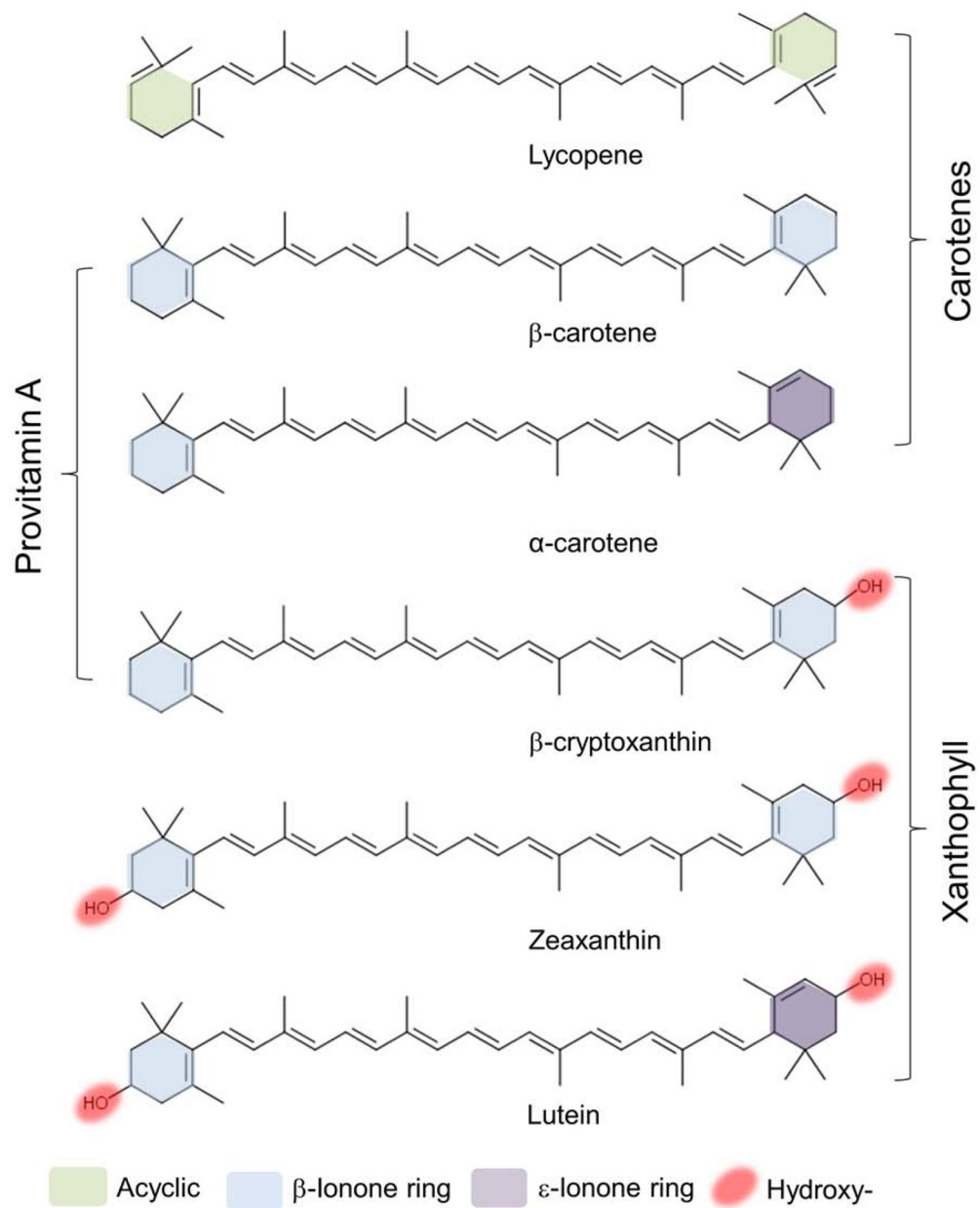
Figure 1.
Structures of the six major carotenoids in human blood.
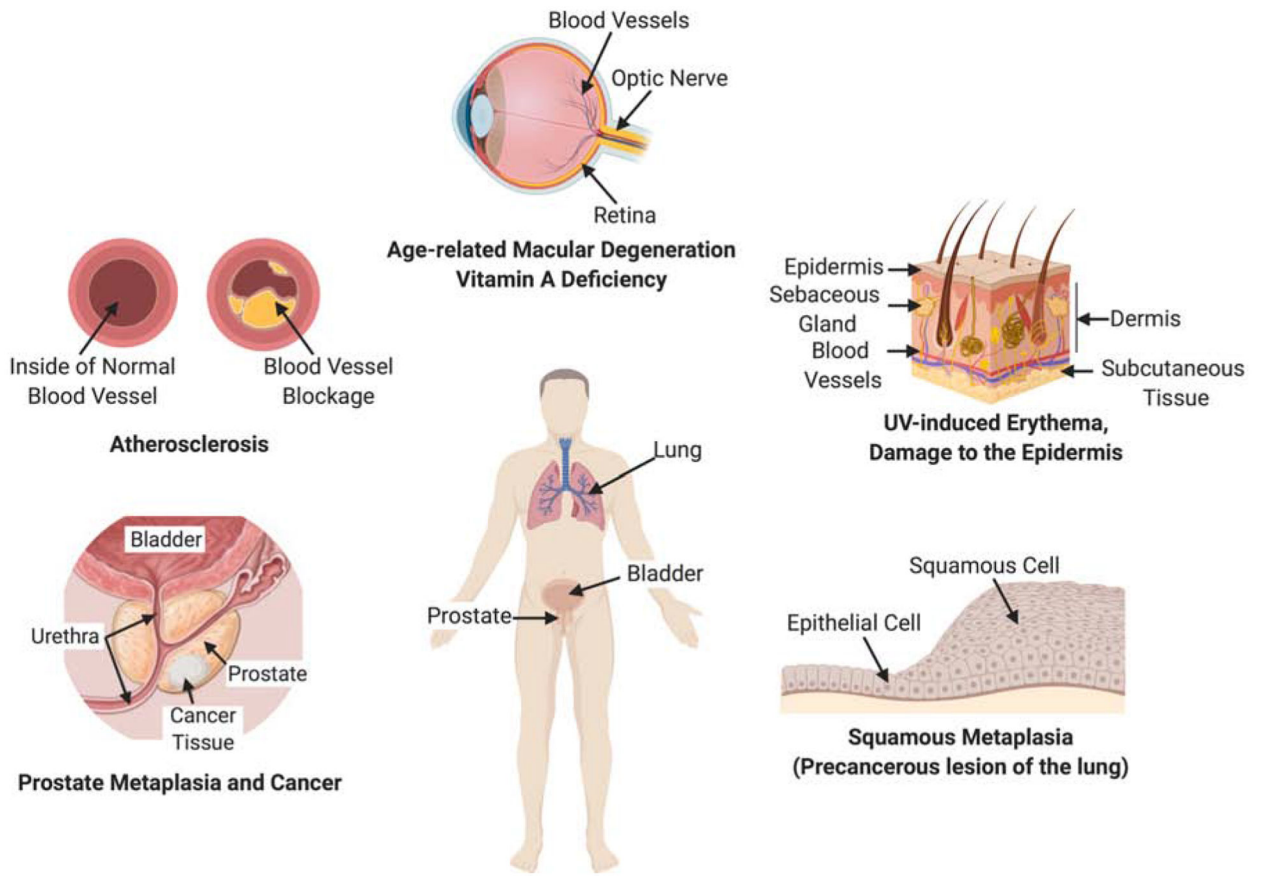
Figure 2.
Health and disease states related to carotenoids.
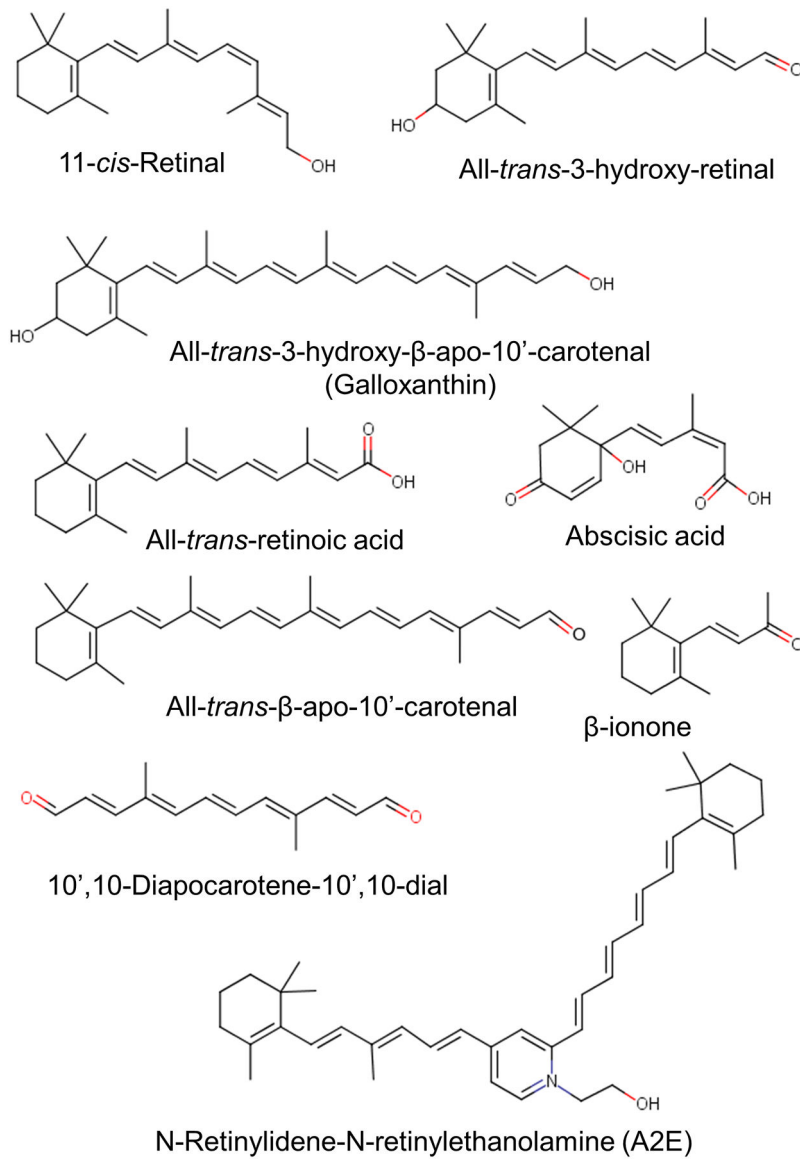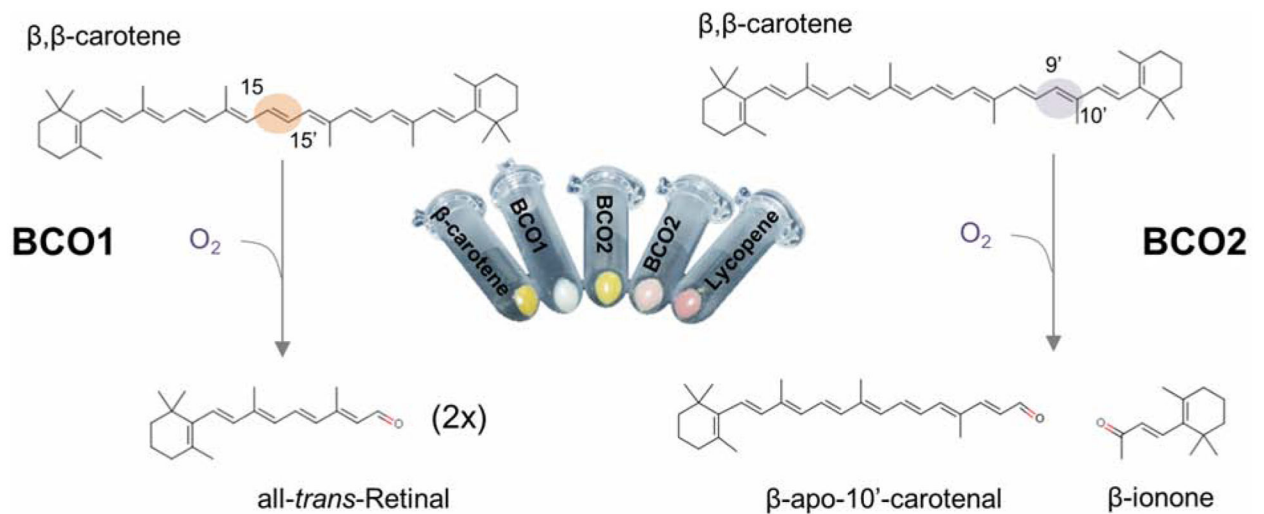
Figure 3.
Structure of various natural apocarotenoids.

Figure 4. Reactions catalyzed by the two mammalian carotenoid cleavage dioxygenases.
Carotenoid conversion by β-carotene-15,15'-dioxygenase (encoded by the *BCO1* gene) (left) and β-carotene-9',10'-dioxygenase (encoded by the *BCO2* gene) (right). The cleavage sites within the carbon backbone of β-carotene are highlighted. In the middle, pellets of bacteria are shown that produce either β-carotene (left) or lycopene (right). Expression of recombinant mouse BCO1 or BCO2 leads to color shifts of the pellets caused by carotenoid conversion to apocarotenoids.
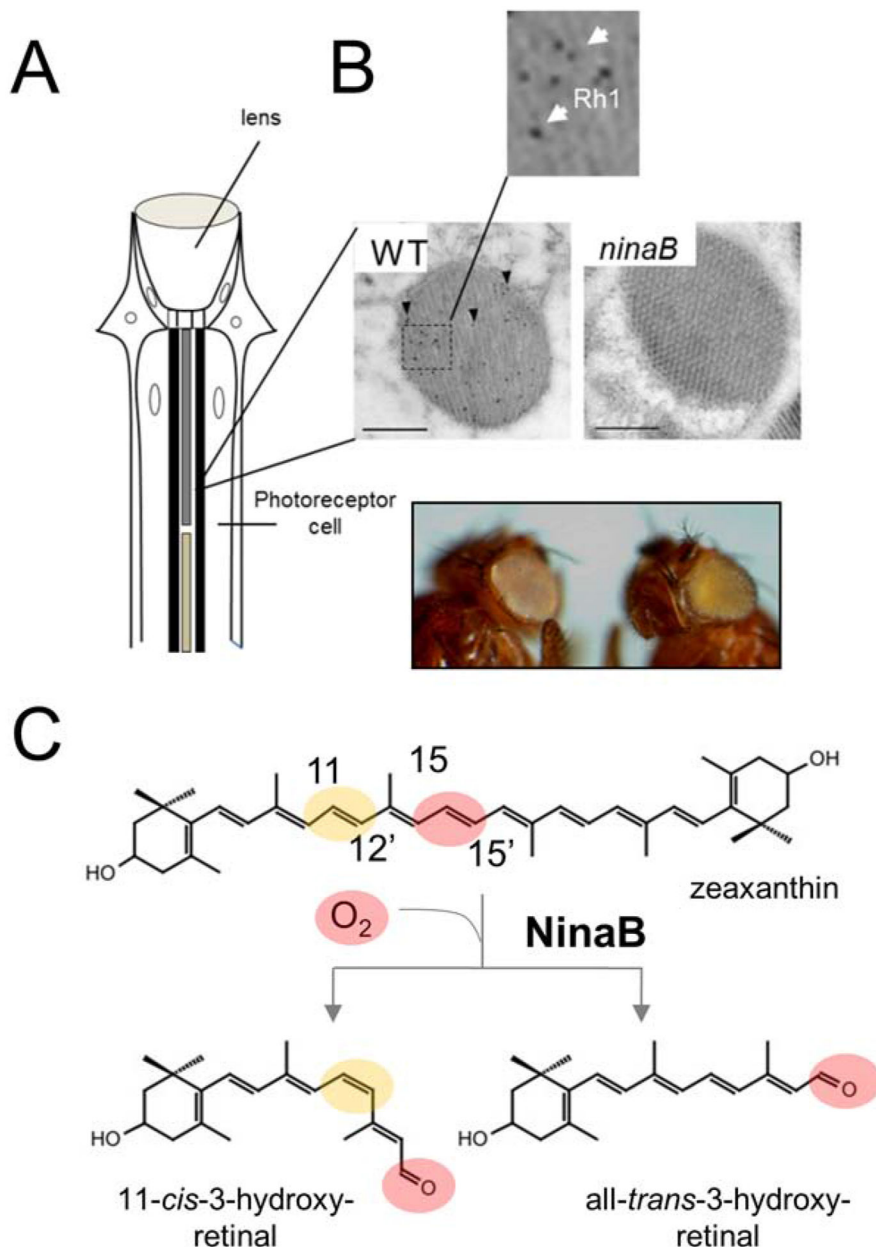
Figure 5. NinaB and chromophore production in arthropods.

(A) Scheme of an omatidium of the insect compound eyes. (B) Electromicrographs of cross sections of rhabdomers immuno-gold stained for rhodopsin 1 (Rh1 encoded by the *ninaE* gene) of wild type and *ninaB* mutant flies. Note that *ninaB*[360d] flies lack Rh1. Photographs of wild type and *ninaB*[360d] mutants on a white eye genetic background. The eyes of *ninaB*[360d] flies appear yellow because of carotenoid accumulation. (C) Reaction catalyzed by NinaB protein with zeaxanthin. The sites for geometric isomerization and oxidative cleavage are respectively highlighted by yellow and red color.
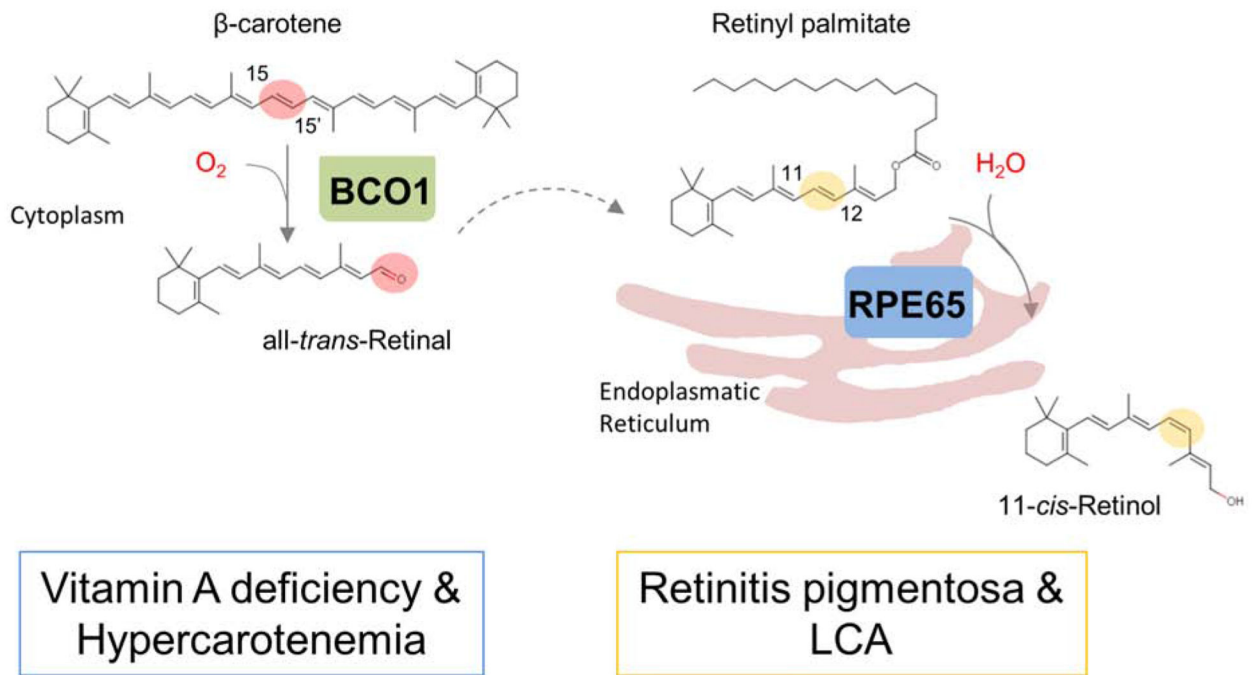
Figure 6. Chromophore production in vertebrates.
In vertebrates, two carotenoid cleavage oxygenases are involved in chromophore production. The β-carotene-15,15'-dioxygenase (encoded by the *BCO1* gene) converts the β-carotene into all-*trans*-retinal. All-*trans*-retinal is further converted to retinyl palmitate. The retinoid isomerase (encoded by the retinal pigment epithelium protein of 65 kDa) splits all-*trans*-retinyl palmitate into 11-*cis*-retinol and palmitate.
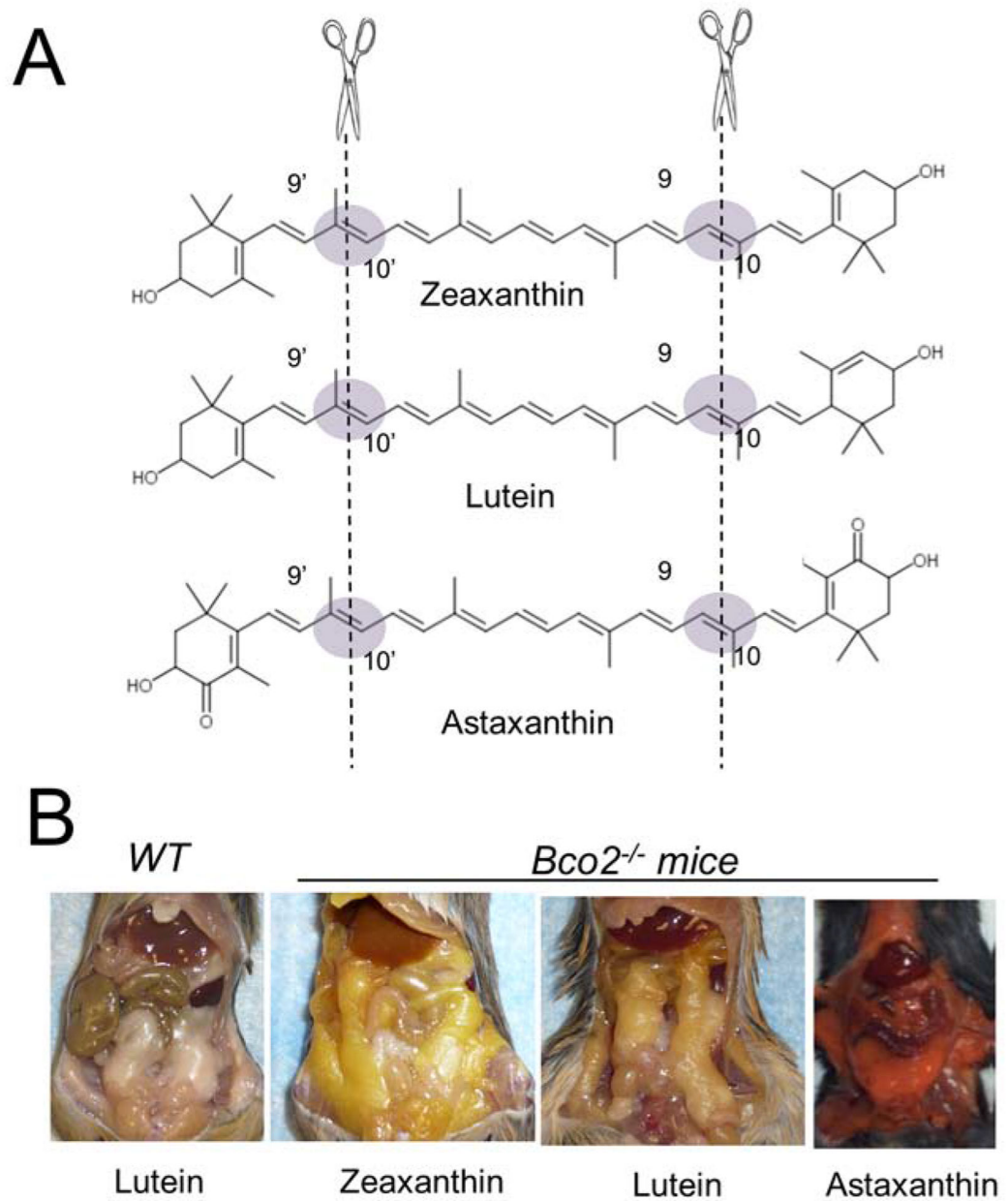
Figure 7. BCO2 mutations and the yellow fat phenotype.
(A) Cleavage sites of β-carotene-9',10'-dioxygenase in zeaxanthin, lutein, and astaxanthin substrates. (B) Yellow and red fat phenotype of *Bco2*⁻ᐟ⁻ mice supplemented with zeaxanthin, lutein, and astaxanthin. Wild type mice do not display fat coloration when supplemented with carotenoids.
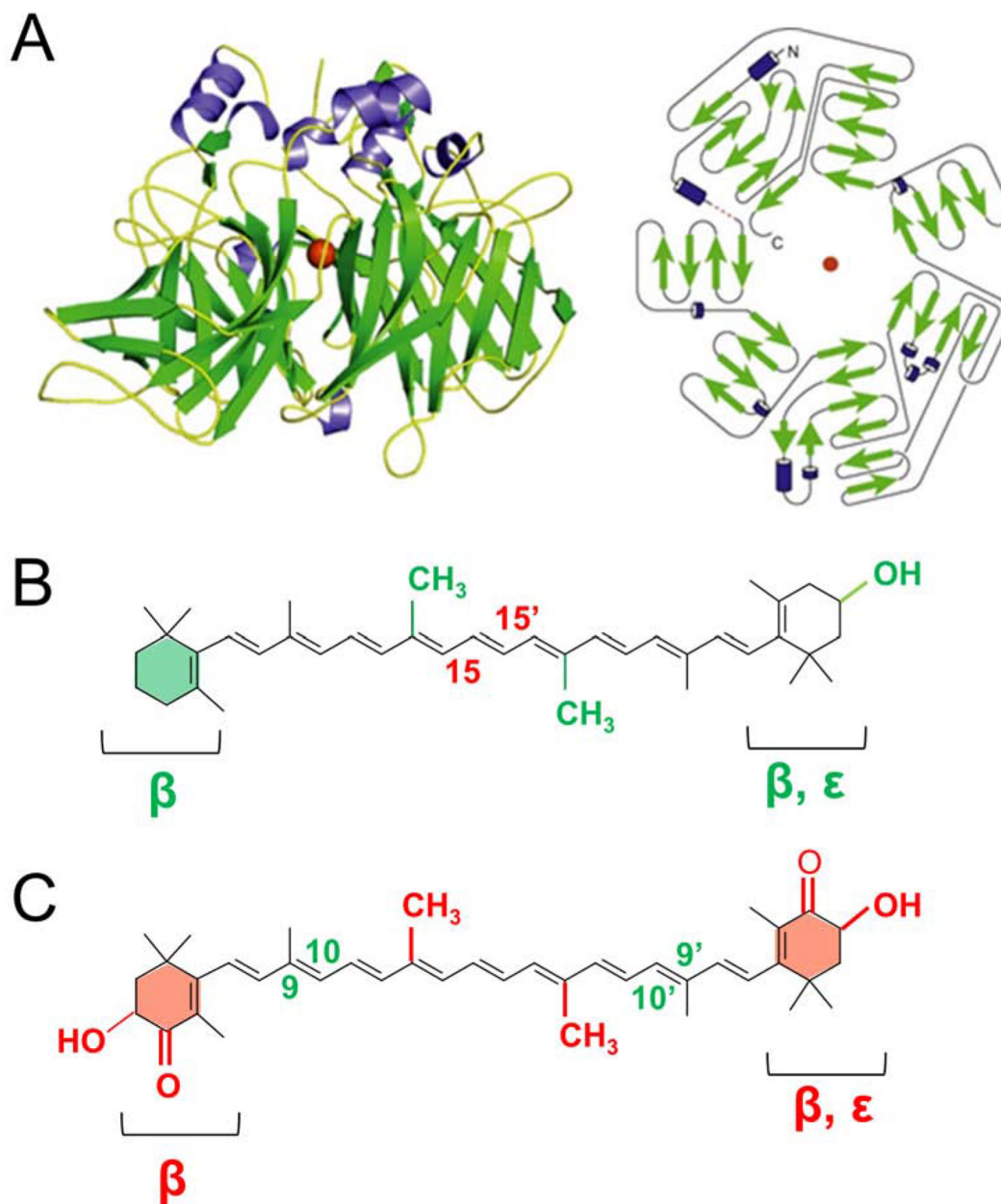
Figure 8. Structure and function of vertebrate CCDs

(A) Crystal structure and topology diagram of bovine RPE65. (PDB accession codes: 3FSN). The ferrous catalytic iron is colored in red. Secondary structural elements consisting of α-helices and β-sheets are colored in blue and in green, respectively. (B) Scheme of substrates of BCO1. The displayed structure highlights in green chemical allowed modifications of BCO1's classical substrate β-carotene. These modifications include 3-hydroxy substitutions of the β-ionone ring, replacement of the β- by an ε-ionone, removal of methyl groups (nor). The central cleavage site at position C15, C15' of BCO1 is indicated in red. Note that BCO1 substrates must contain at least one none-substituted β-ionone ring. (C) Scheme of substrates of recombinant mouse BCO2. The structure highlights in red the allowed chemical modifications of BCO2's classical substrate BC. These modifications

include 3-hydroxy substitutions and 4-oxo-substitutions of the β-ionone ring, replacement of the β- by an ɛ-ionone, or removal of methyl groups (nor). The eccentric cleavage sites at positions C9, C10 and C9, C10' of BCO2 are indicated in green. Note that BCO2 cleaves substrates with assorted ionone ring site modifications.
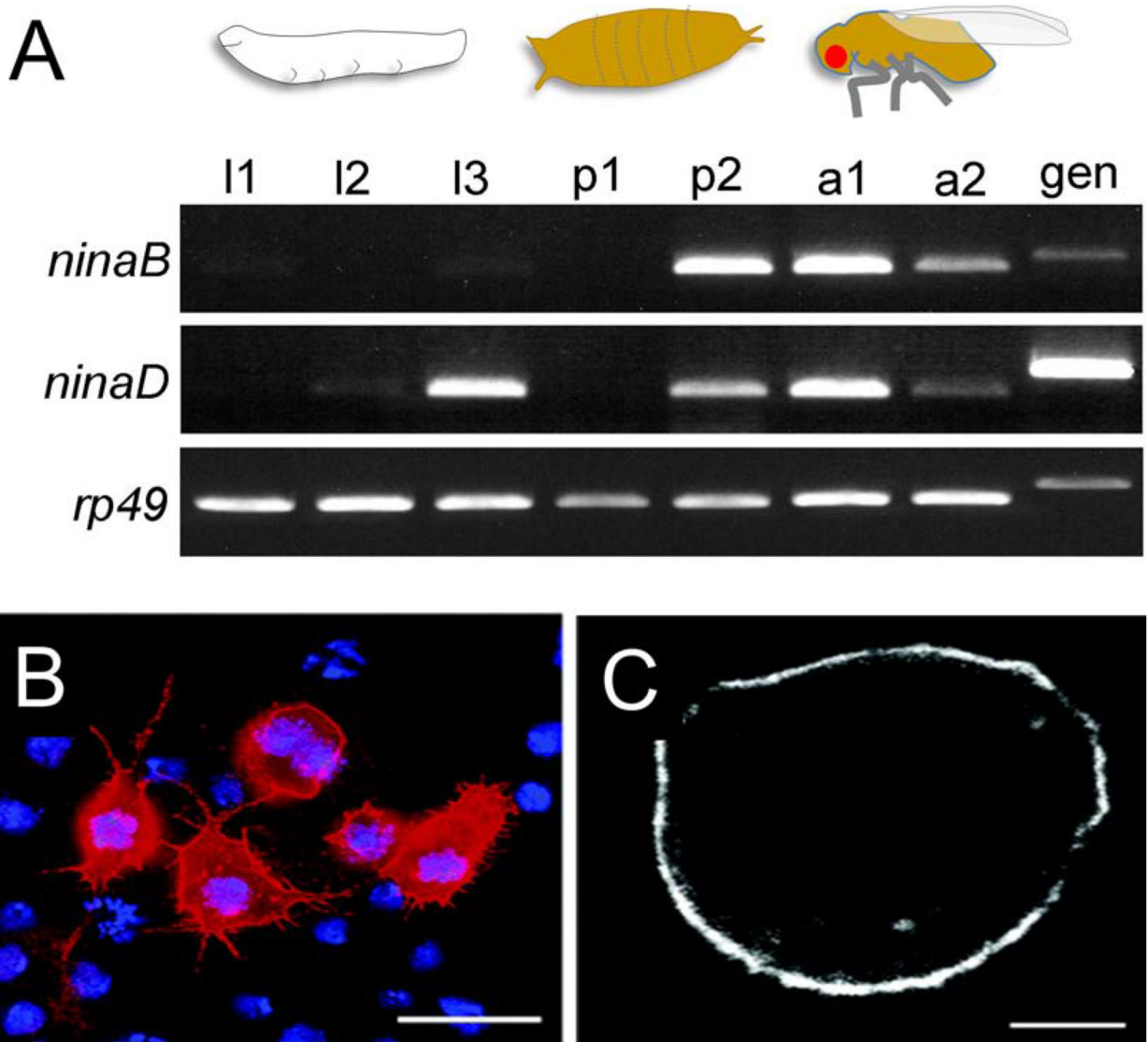
Figure 9. NinaD and carotenoid transport.

(A) Temporal expression patterns of *NinaD* and *NinaB* during the flies' life cycle. *NinaD* is expressed at late larval stages and helps rendering carotenoids available from the chow. *NinaB* expression increases at late pupal stages timely coinciding with rhodopsin synthesis in the compound eyes. l1–3, first, second and third instar larval stages; p1, early pupa; p2, late pupa; a1, adult (< 3 days old); a2, adult (10 days old), gen, genomic DNA control. (B, C) Immunocytochemistry of transiently transfected S2 cells expressing *ninaD-I*. Panels B shows an epifluorescence image showing labeling of NinaD-I, which was detected with an antibody raised against the V5 epitope of the recombinant NinaD fusion protein and Cy3-conjugated secondary antibody (red). Cell nuclei are stained with DAPI (blue). Scale bar is 10 μm. Panels C shows a confocal image depicting the immunofluorescence labeling of NinaD-I in one optical section that is approximately 0.26 μm thick. Scale bars are 2.5 μm.

Mixed micelles
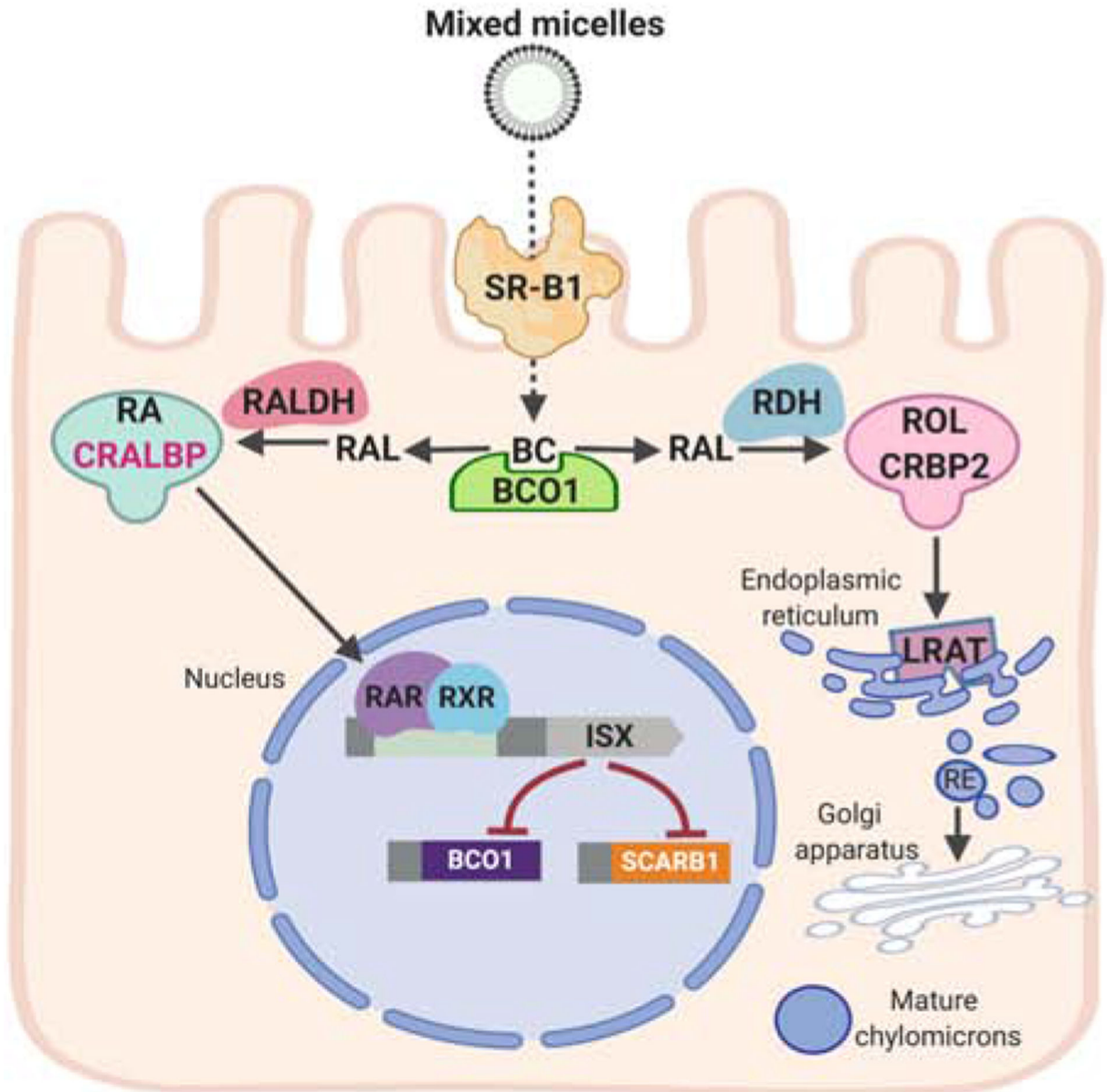
Figure 10. A diet-responsive regulatory network controls intestinal vitamin A production and carotenoid absorption.

β-Carotene (BC) absorption is facilitated by scavenger receptor class B type 1 (SR-B1). The conversion of BC by β-carotene-15,15'-dioxygenase (BCO1) is an important branching point in retinoid metabolism. The primary product retinaldehyde (RAL) is reduced to retinol (ROL) by retinal dehydrogenases (RDH) in a process that involves cellular retinol-binding protein (CRBP2). ROL is the converted to retinyl esters (RE) by lecithin: retinol acyl transferase (LRAT) and packed in chylomicrons for body distribution. Some RAL is also converted to retinoic acid (RA) by retinal dehydrogenase (RALDH). RA binds to retinoic acid receptors (RARs) that in conjunction with retinoid X receptors (RXR) regulate the

expression of intestine specific homeobox (ISX) transcription factor. ISX is a transcriptional repressor of the genes encoding SCRAB1 and BCO1.
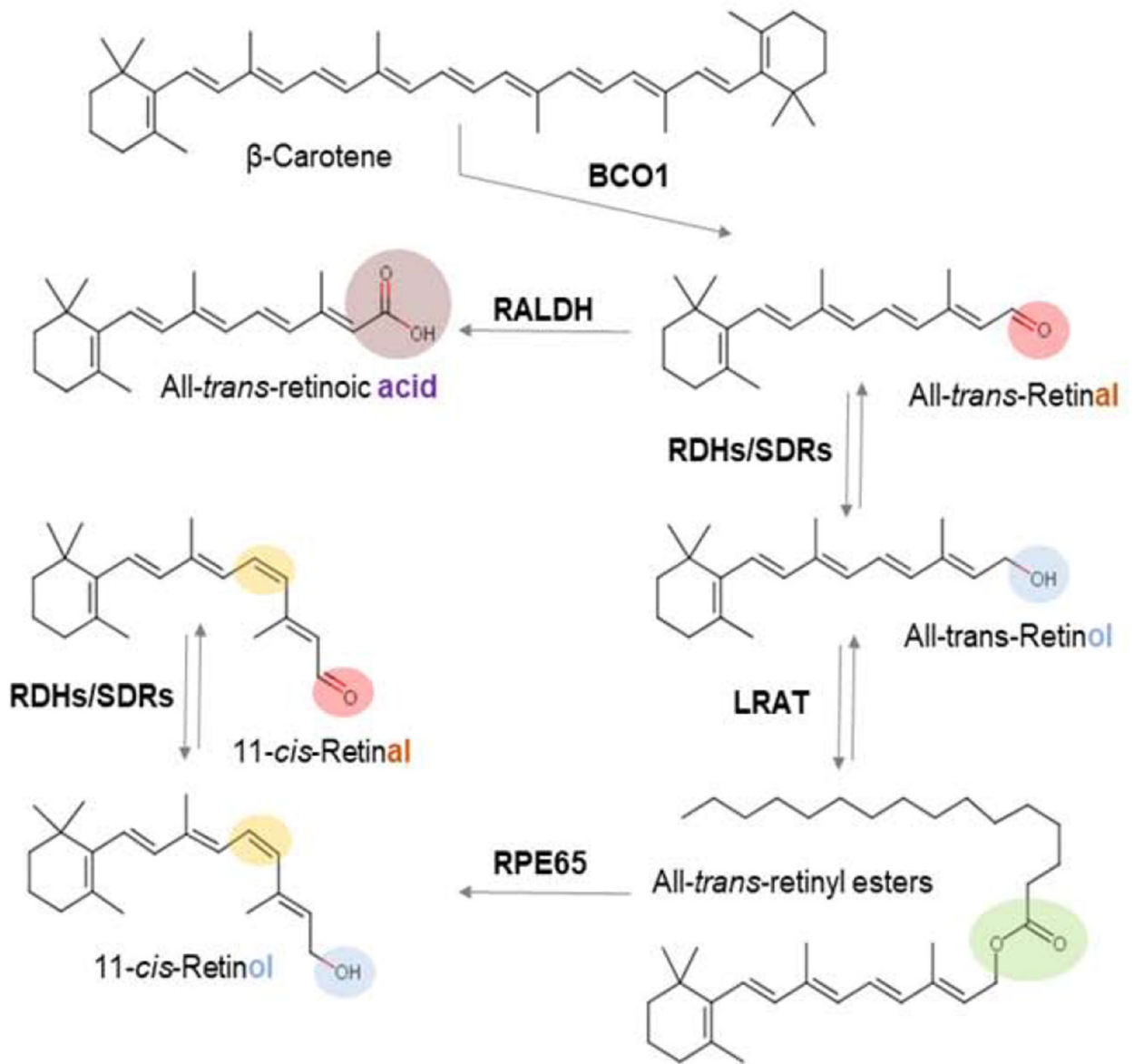
Figure 11. Overview of mammalian vitamin A metabolism.
The different functional groups of retinoids are indicated by a color code (red, aldehyde; blue, alcohol; green, ester; purple, acid). The 11-*cis*-retinoid diastereoisomer is indicated by yellow color. Major retinoid metabolizing enzyme classes (see main text) catalyzing the chemical transformations of retinoids are highlighted.
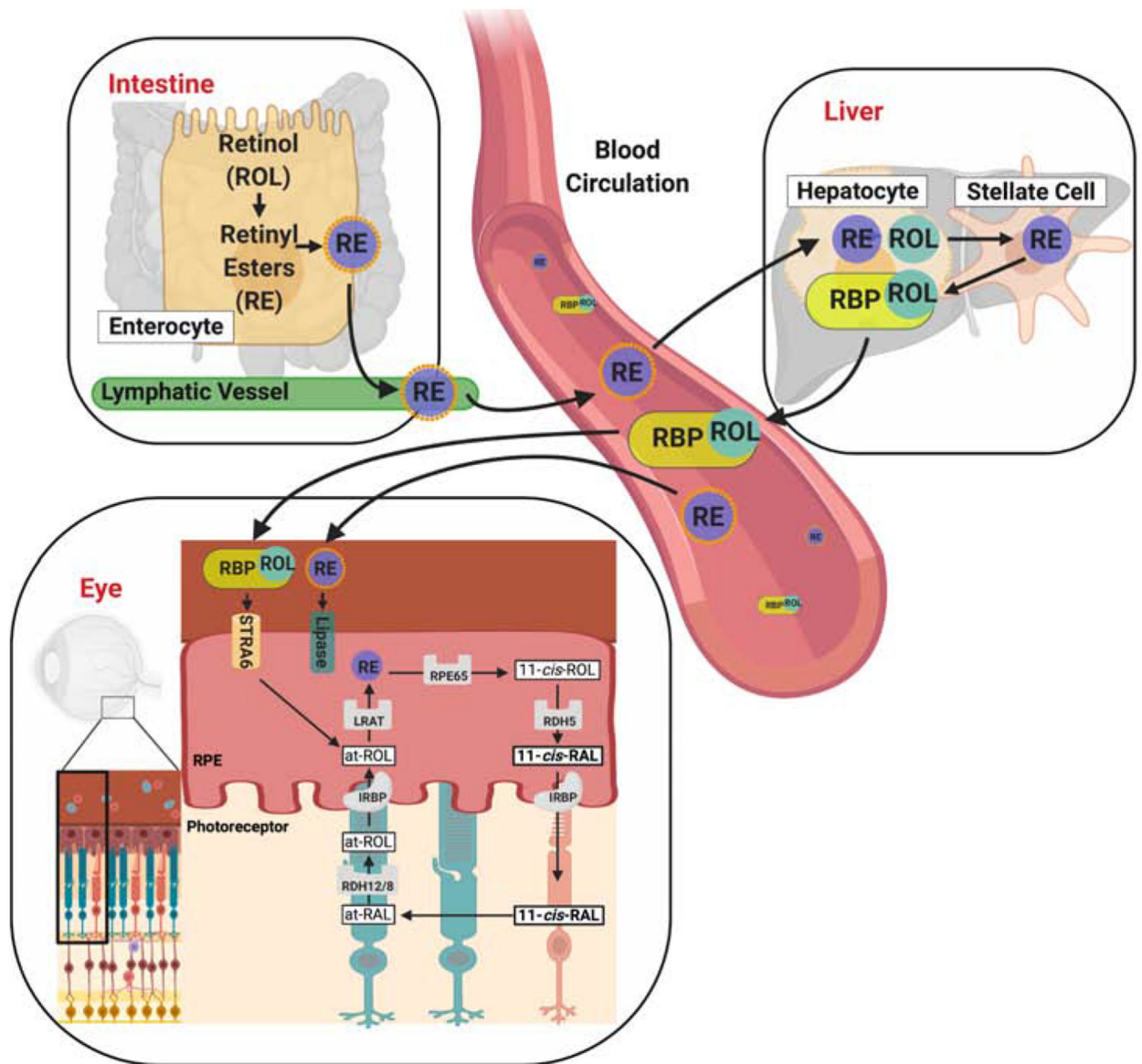
Figure 12. Transport of vitamin A throughout the body.
Carotenoids such as β-carotene are absorbed in brush border cells in the intestine, where
they are converted into retinyl esters (RE), packaged into chylomicrons, and released into
the circulation. The RE in chylomicrons can either be delivered to peripheral cells and taken
up in a lipoprotein lipase-mediated process or be transported for receptor-mediated
endocytosis and storage to the liver. In the liver, stored RE are converted back to retinol
which binds to serum retinol-binding protein (RBP, encoded by the RBP4 gene). The retinol-
RBP complex is released into the circulation. Vitamin A is taken up from holo-RBP in a
STRA6-mediated transport process. In the retina pigment epithelium (RPE) of the eyes,
vitamin A is esterified during uptake and converted to chromophore through the visual cycle.
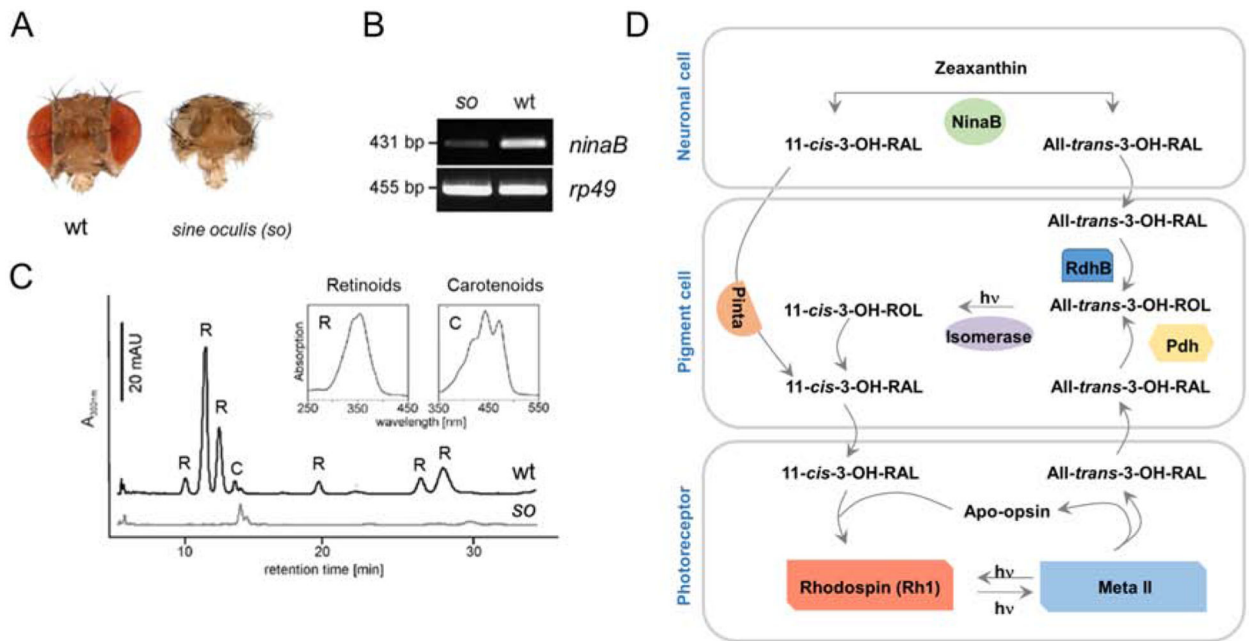RAL, retinal, ROL, retinol, RDH, retinal dehydrogenase

Figure 13. The insect visual cycle.

(A) Heads of wild type and *sine oculis* flies, (B) Semiquantitative RT-PCR analysis for *ninaB* expression with total RNA preparations from the heads of adult *sine oculis* and wild-type flies. (C) HPLC traces obtained from lipid extracts of 70 mg of wild-type (*upper trace*) and *sine oculis* (*lower trace*) fly heads. Different retinoids and carotenoids are indicated by *numbers. Peaks*: *1*, 9/13-*cis*-3-hydroxy-retinal oxime (*syn*); *2*, all-*trans*- (*anti*) and 11-*cis*-3-hydroxy-retinal oxime (*syn*); *3*, all-*trans*-3-hydroxy-retinal oxime (*syn*); *4*, zeaxanthin/lutein; *5*, all-*trans*-3-hydroxy-retinol; *6*, 9-*cis anti*-3-hydroxy-retinal oxime; *7*, 13-*cis*-3-hydroxy-retinol; and *8*, 11-*cis*-(*anti*)-3-hydroxy-retinal oxime. Insets show the spectra of *peak 3*, all-*trans*-retinal oxime (*syn*) and *peak 4*, zeaxanthin. (D) Scheme of the fly's chromophore metabolism.
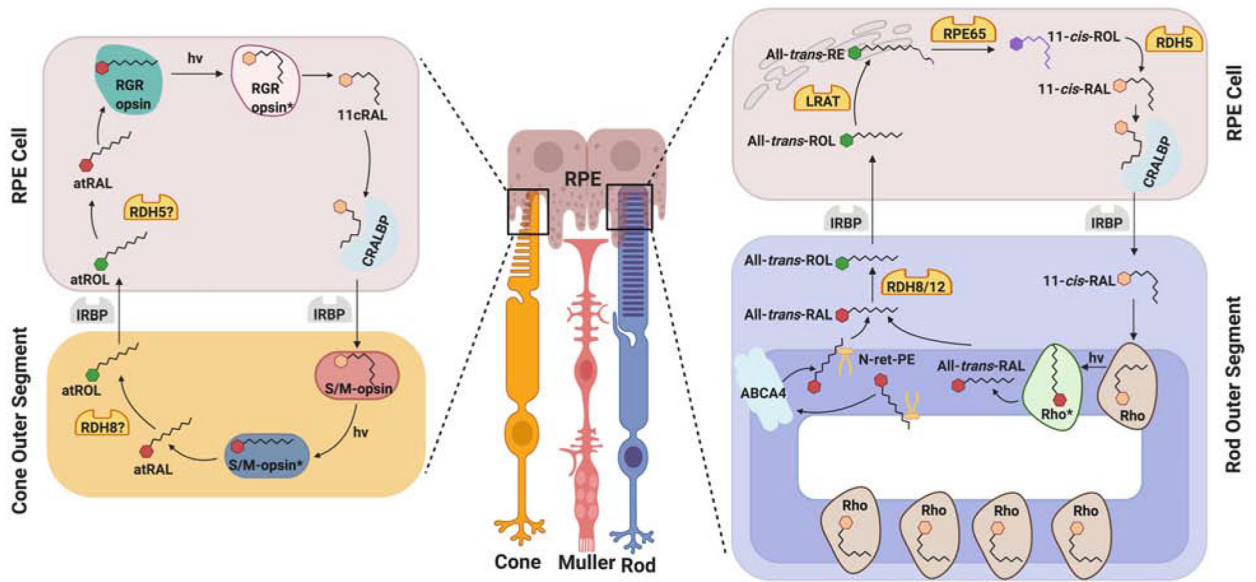
Figure 14. The vertebrate visual cycle.
(central) Simplified schematic overview of the arrangement of retinal pigment epithelium cells, rod and cone photoreceptor cells, and Müller glial cells. (right) Biochemical key steps of the classical visual cycle. (left) Photic RGR-dependent visual cycle. N-ret-PE, N-retinylidene-phoshatidyl-ethanolamine, RAL, retinal, RE, retinyl ester, Rho, rhodopsin, ROL, retinol.
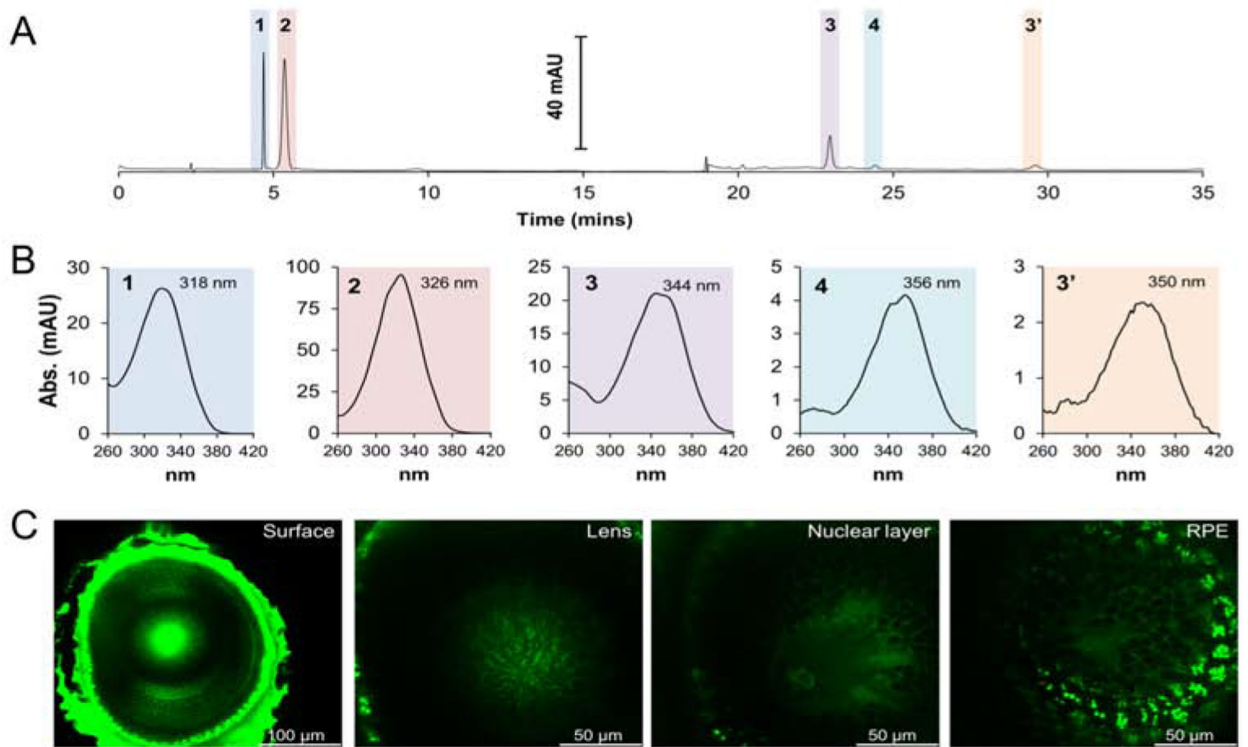
Figure 15. 11-*cis*-retinyl esters exist in the retinal pigment epithelium (RPE) in zebrafish larvae.
(A) Representative HPLC chromatogram at 325 nm of lipophilic extracts of 5-days post fertilization zebrafish larvae. (B) Spectral characteristics of extracted retinoids from zebrafish larvae. (C) *In vivo* two-photon microscopy images of different layers of the zebrafish larval eye. Retinosomes were exclusively detected in the RPE (far right image). 1, 11-*cis*-retinyl esters (11-RE); 2, all-*trans*-retinyl esters (at-RE); 3, *syn* 11-*cis*-retinal oxime (*syn* 11-RAL); 4, *syn* all-*trans*-retinal (all-RAL); 3', *anti* 11-*cis*-retinal oxime (*anti* 11-RAL).
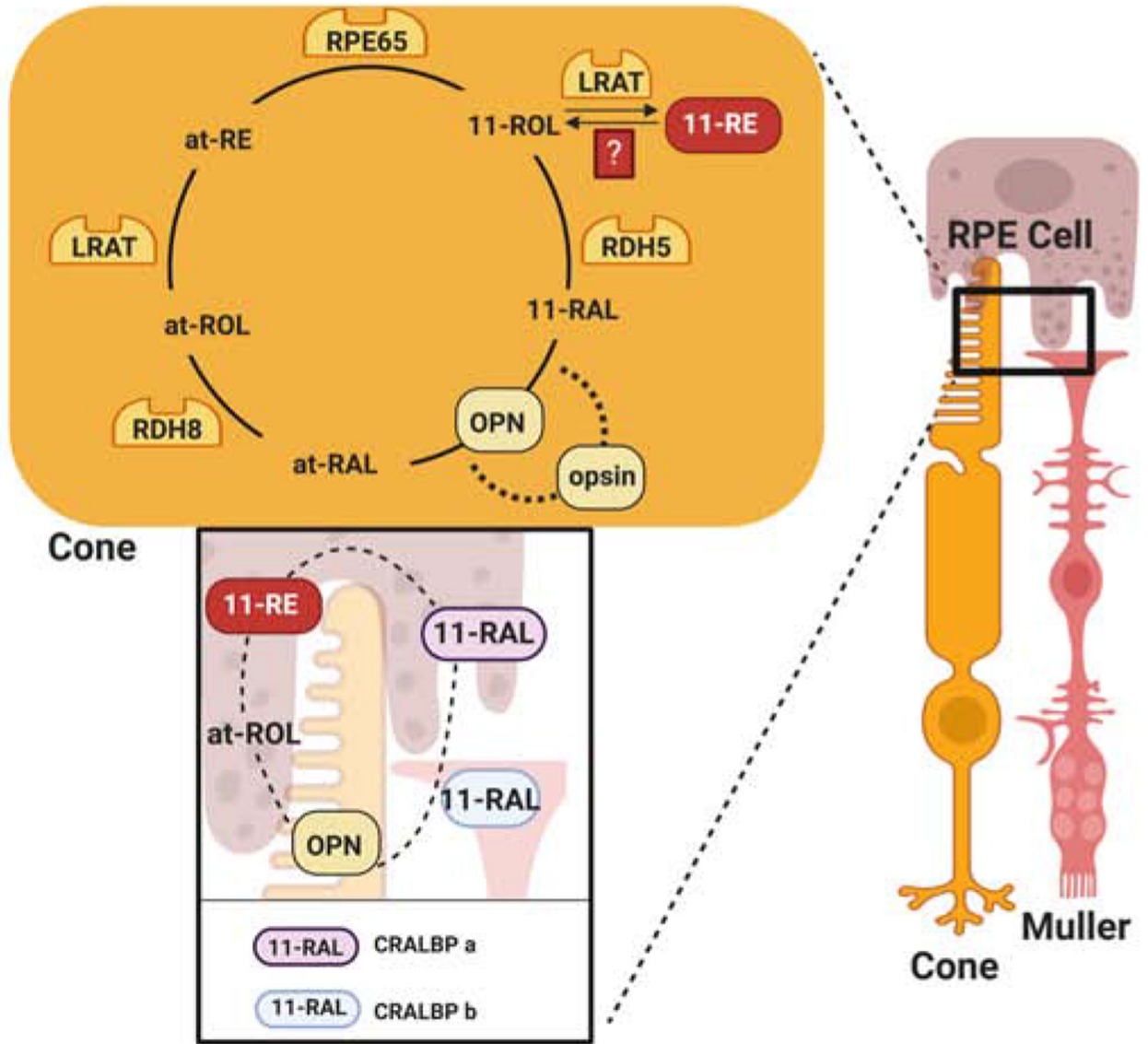
Figure 16. Proposed 11-*cis*-retinyl ester cycle of the zebrafish eyes.
Within the RPE, under dark conditions, RPE65 forms 11-*cis*-retinol in a canonical fashion. Subsequently, this 11-cisretinol is esterified by LRAT for storage in retinosomes in the form of 11-cis-retinyl esters. An unidentified light-dependent hydrolase hydrolyzes 11-cis-retinol into 11-cis-retinol when demand for the chromophore would arise. The newly formed 11-cis-retinol would be oxidized to 11-cis-retinal and shuffled from the RPE to Müller glia cells via a CRALBPa and CRALBPb-dependent mechanism. In the cones, 11-cis-retinal binds to cone opsins to form the cone opsin pigment molecules for use in phototransduction. Recycling of the released chromophore, all-trans-retinal will proceed in a canonical fashion. 11-RAL, 11-*cis*-retinal; at-RAL, all-trans-retinal, 11-RE, 11-*cis*-retinyl ester; at-RTE, all-*trans*-retinyl ester. 11-ROL, 11-cis-retinol; at-ROL, all-*trans*-retinol.